CHARACTERISING THE ROLE OF SUBSTANCE P

IN ACUTE ISCHAEMIC STROKE

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CHAPTER 5:

COMBINATION THERAPY OF AN NK_1 RECEPTOR ANTAGONIST AND TISSUE PLASMINOGEN ACTIVATOR IN ISCHAEMIC STROKE

5.1 Introduction

The results of the previous chapters demonstrate that SP release is a feature of ischaemic stroke and that it is associated with profound BBB dysfunction, cerebral oedema, functional deficits and histological abnormalities. Subsequent administration of the NK_1 receptor antagonist, NAT, was highly efficacious in ameliorating these post-stroke sequelae. Hence, antagonism of the SP pathway may provide a novel avenue for the treatment of ischaemic stroke. Current stroke treatment incorporates the use of thrombolytics, and any potential therapeutic strategy must therefore be suitable for use in conjunction with such thrombolytic agents. The current study investigates the combined use of an NK_1 receptor antagonist with a thrombolytic agent.

Tissue plasminogen activator (tPA) is a thrombolytic agent that converts inactive plasminogen to plasmin and hence is able to dissolve blood clots impeding blood flow (Longstaff and Thelwell, 2005). Reperfusion of the ischaemic territory is desirable as it may salvage compromised but viable tissue that would otherwise die (Yang and Betz, 1994; Aronowski et al., 1997; Schaller and Graf, 2004). However, restoration of blood flow may also cause reperfusion injury, as re-delivery of oxygen to the tissue leads to free radical formation, exacerbating injury and potentially leading to infarct extension (Przyklenk and Kloner, 1989).

The use of tPA was approved for administration within 3 h of stroke onset in 1996 by the United States Food and Drug Administration, following results of the NINDS trial demonstrating a reduction in death and dependency after stroke by tPA (Kwiatkowski et al., 1999). In the NINDS trial, 624 patients with acute ischaemic

stroke were treated with tPA (0.9 mg/kg to a maximum dose of 90 mg) or placebo within 3 h of symptom onset. A favourable outcome at 3 months post-stroke, of near or complete recovery, was observed in 31-50% of tPA-treated patients compared to 20-38% of placebo-treated patients. Mortality rates were comparable at 3 months and 1 year post-stroke. The major risk associated with treatment was symptomatic brain haemorrhage, which occurred in 6.4% of cases in the tPA group and only 0.6% in the placebo group. Overall, early recanalisation of the arteries was able to salvage endangered tissue and favourably affect outcome. Despite these findings, there has been much criticism of the NINDS trial, although recent re-analysis of the trial confirmed the original observations. Similarly, the European co-operative acute stroke study (ECASS) trials, ECASSI and ECASSII, reported that patients treated with tPA within 3 h did benefit from the treatment, and that death and disability was reduced compared to the placebo group (Hacke et al., 1995; Hacke et al., 1998). Nevertheless, apprehension regarding tPA therapy remains, with the increased risk of haemorrhagic complications central to such concerns.

In the blood, tPA functions as a fibrinolytic agent, however in the CNS it may mediate events associated with cell death (Polavarapu et al., 2007). Normally, endogenous tPA circulates in the bloodstream as a complex with plasminogen activator inhibitor (PAI-1). During tPA administration following stroke, exogenous tPA may saturate PAI-1, thereby allow free tPA to circulate in the bloodstream, potentially causing BBB damage with subsequent cerebral oedema formation and neuronal death (Zhang et al., 2002). Tsirka and colleagues were the first group to report on the neurotoxicity of tPA (Tsirka, 1997), demonstrating that mice lacking tPA were resistant to excitotoxic mediated hippocampal neuronal degeneration.

Subsequent studies have shown that exogenous tPA (1mg/kg) significantly increases infarct size following stroke (Wang et al., 1998). Nonetheless, other groups have reported a reduction in infarct volume following administration of exogenous tPA (Yi et al., 2004), or by tPA deletion in knockout models (Nagai et al., 1999; Wang et al., 1998). More recent studies have confirmed that tPA administration may have deleterious effects in addition to its' favourable effects as a thrombolytic agent (Kaur et al., 2004; Wang et al., 1999) with the intravascular effects of tPA being beneficial, and the extravascular effects detrimental (Kaur et al., 2004; Zhang et al., 2002). The negative consequences of tPA therapy include cerebral oedema, haemorrhage and exacerbation of the ischaemic insult (Kaur et al., 2004). These deleterious effects observed following tPA administration may be, in part, explained by the activation of matrixmetalloproteinases (MMP). This family of proteinases have the ability to degrade components of the extracellular matrix including collagen, fibronectin, laminin and proteoglycans (Rosenberg et al., 1998). The MMPs normally play important physiologic roles in processes such as embryonic remodelling, wound healing and angiogenesis. However, the uncontrolled expression of MMPs can lead to tissue injury. Activation of MMPs has a direct effect on the integrity of the extracellular matrix and basal lamina with integral structural proteins being degraded. Such disruption of the vascular architecture leads to increased BBB permeability, oedema formation and haemorrhagic transformation (Kaur et al., 2004), all of which may further exacerbate ischaemic damage and lead to extension of the lesion. Increases in MMP-9 levels have been detected following experimental and clinical stroke (Rosenberg et al., 1998; Ning et al., 2006).

In the clinic, the application of tPA therapy is limited and it has been reported that less than 5% of all stroke patients receive tPA, mainly due to the short therapeutic window within which it must be administered (Marler and Goldstein, 2003). Administration of tPA beyond the 3 h window is associated with an increased risk of haemorrhagic complications, worsened outcome and death. Spontaneous haemorrhagic transformation occurs in up to 30-40% of cases of ischaemic stroke, and is more prevalent and severe following fibrinolysis with tPA (Asahi et al., 2000; Jaillard et al., 1999; Larrue et al., 2001; Warach and Latour, 2004). Hence, there is a genuine need for an intervention that may be combined with tPA to reduce neurotoxicity, decrease the risk of haemorrhage and death, reduce reperfusion injury, amplify the neuroprotective effect and potentially increase the therapeutic window. As such, the aim of the present study was to characterise the effect of the combined therapy, comprising a NK_1 receptor antagonist and tPA, on BBB status, functional outcome and histological abnormalities following ischaemic stroke.

5.2 Study Design

Animals (n=64) were randomly assigned to sham and treatment groups. Following stroke (as described in Chapter 2.2.2), animals received either equal volume of saline vehicle or the drug treatments NAT (25 μmoles/kg) and tPA (1mg/kg), either alone or in combination, administered via the tail vein immediately following the onset of reperfusion. Drugs were prepared and administered as described in chapter 2.

5.2.1 Histology Study

Following perfusion at the pre-determined time–point, brains were processed for immunohistochemistry as described in chapter 2. Sections were then stained for H&E, SP, APP, GFAP and ED-1 and then assessed using light microscopy. Sections were also stained with FJC and assessed using fluorescence microscopy.

5.2.2 Blood Brain Barrier Study

As previously described in detail elsewhere (Chapter 2), Evan's Blue dye was used to assess the permeability of the BBB. The experiment was divided up into 2 parts, one being the effects of tPA on barrier integrity in naïve animals and the other being the effects of tPA on barrier integrity in stroke animals. In both cases, the efficacy of NAT to reduce barrier permeability was assessed.

5.2.3 Functional Outcome

Commencing at 24 h post-reperfusion, animals were assessed using the rotarod, bilateral asymmetry test, open field, modified neuroseverity score and the angleboard as described in detail in Chapter 2. Functional outcome testing was carried out daily for a 7 d period following stroke.

5.2.4 Statistical Analysis

The BBB data was analysed using a one-way ANOVA followed by individual Newman-Keuls post-hoc tests. The Neuroscore data was analysed using the non-parametric Kruskall-Wallis test followed by individual Dunn's Multiple Compartison tests. The parametric data was analysed using two-way ANOVA followed by individual Bonferroni post-tests. The mortality data was analysed

using Fisher's exact test. All data are expressed as mean ± SEM, with the exception of the neuroscore data, which is expressed as the median. A p value of 0.05 was considered significant.

5.3 Results

5.3.1 Mortality

A non-significant increase in mortality was observed in the tPA group, compared to either the NAT or NAT/tPA groups (Figure 5.1). The survival rate in the tPA group was only 45% compared to 83% and 61% in the NAT and NAT/tPA groups respectively. The mortality rate was comparable to the 50% observed in the vehicle group. Power analysis revealed that for such differences to be statistically significant, group sizes would need to be at least trebled. A decrease in survival rate following tPA administration has previously been reported (Pfefferkorn and Rosenberg, 2003).

### 5.3.2 Effect of tPA on mortality and incidence of intracerebral haemorrhage

An increased incidence of ICH was observed in tPA-treated animals that died (Figure 5.2), with 77% of animals suffering an ICH. The incidence of ICH in the vehicle, NAT and NAT/tPA groups was very low, being 9%, 5% and 21% respectively. Although specific differences amongst groups were unable to be determined, a significant effect of treatment was apparent ($p<0.001$). Haemorrhagic transformation is a known complication of tPA therapy and an increased incidence of ICH has previously reported following tPA administration (Asahi et al., 2000; Jaillard et al., 1999; Larrue et al., 2001; Warach and Latour, 2004).
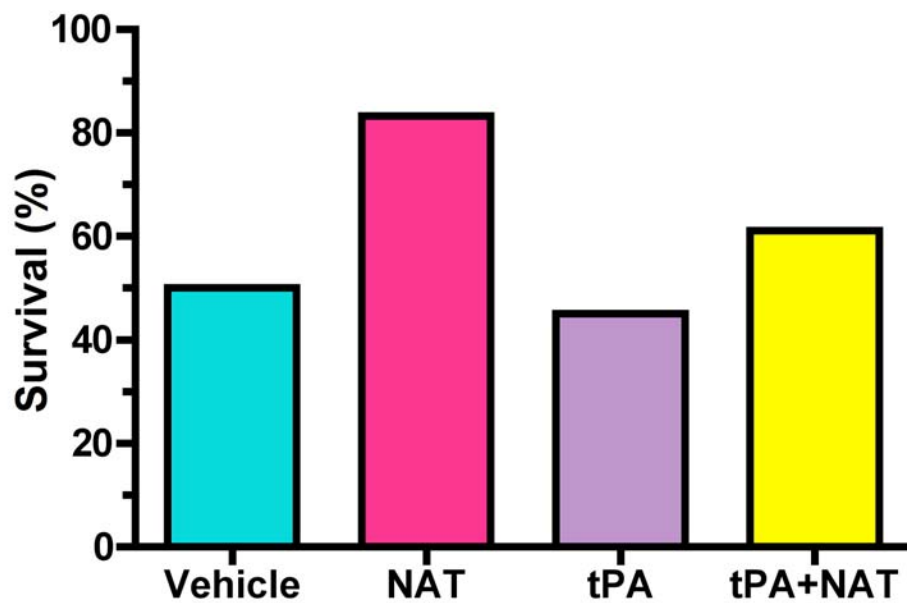
Figure 5.1 NAT/tPA combination therapy. Percentage survival at 7 d post-stroke.

There were no significant differences in 7 d survival between the treatment groups. However, there was a trend towards improved survival in NAT-treated animals and a trend towards a decreased survival in the vehicle- and tPA-treated animals (Vehicle n=12; NAT n=7; tPA n=17; NAT/tPA n=13).
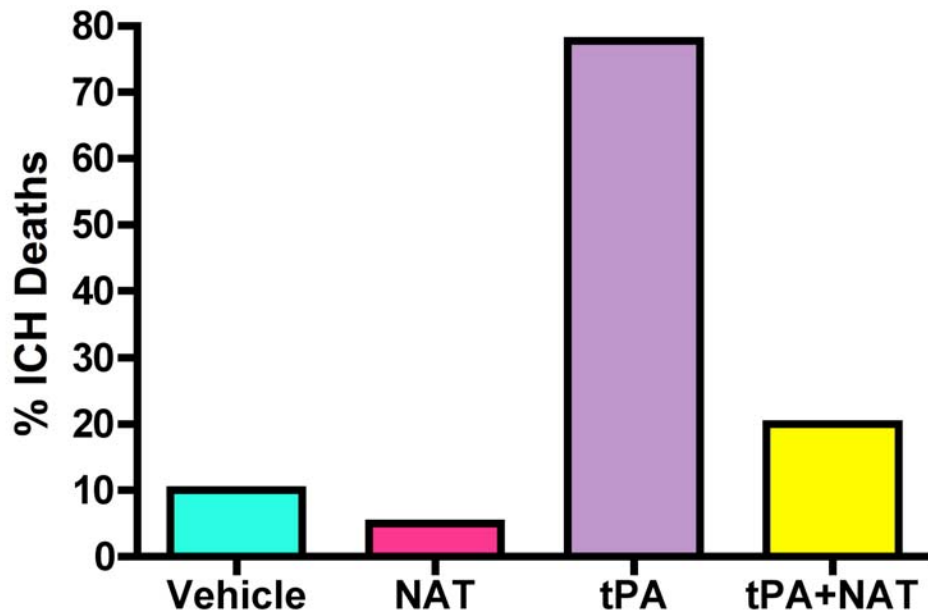
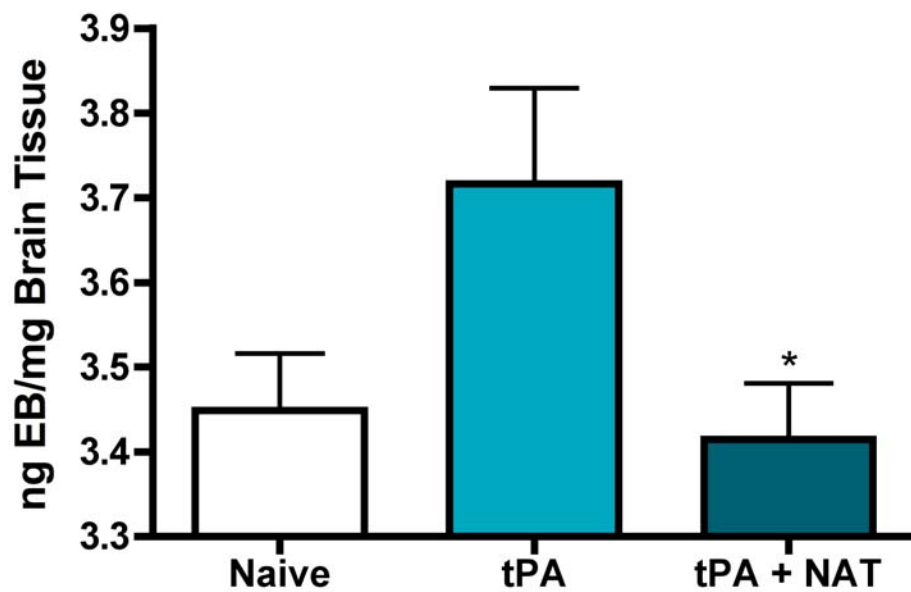**Figure 5.2 NAT/tPA combination therapy. Deaths attributable to intracerebral haemorrhage (ICH).**

The prevalence of ICH as a cause of death within the vehicle and NAT groups was low. However the incidence of ICH was markedly increased in the tPA group. A reduction in ICH risk was associated with the combination therapy of tPA and NAT.

5.3.3 The effects of tPA on the blood brain barrier in naïve animals

Naïve animals were administered tPA to determine the effect of tPA on the integrity of the BBB. The EB extravasation in naïve animals was 3.449 ng EB/mg brain tissue. Administration of intravenous tPA to naïve animals resulted in opening of the BBB (Figure 5.3), such that permeability was 3.716 ng EB/mg brain tissue. Subsequent administration of NAT significantly reduced the tPA-induced barrier opening ($p < 0.05$), such that EB extravasation was 3.415 ng EB/mg brain tissue. tPA-induced opening of the BBB after i.c.v administration has previously been reported (Yepes et al., 2003), with the current study confirming that such increased permeability also occurs following i.v administration.

5.3.4 The effect of tPA and NAT on blood brain barrier breakdown following stroke

The EB extravasation in sham animals was 4.174 ng EB/mg brain tissue. Following stroke, there was a significant increase ($p < 0.001$) in the permeability of the BBB to EB in vehicle animals to 5.940 ng EB/mg brain tissue, as compared to sham animals (Figure 5.4). However, treatment with NAT significantly reduced ($p < 0.001$) BBB permeability to 3.643 ng EB/mg brain tissue, levels comparable to sham levels ($p > 0.05$). Administration of tPA at the onset of reperfusion significantly reduced BBB permeability as compared to vehicles. However, such levels were still significantly higher ($p < 0.001$) than NAT-treated animals at 4.975 ng EB/mg brain tissue. The combination therapy of NAT/tPA reduced BBB permeability to 0.003812 ng EB/mg brain tissue, levels comparable to shams ($p > 0.05$).

Figure 5.3 NAT/tPA combination therapy. Effect of tPA administration on EB extravasation in naïve animals.

Administration of tPA to naïve animals resulted EB extravasation into the brain tissue, indicative of BBB opening. Administration of NAT significantly reduced ($p<0.05$) the tPA-induced BBB opening (*denotes $p<0.05$ versus tPA; Naïve n=5; tPA n=6; tPA+NAT n=8).
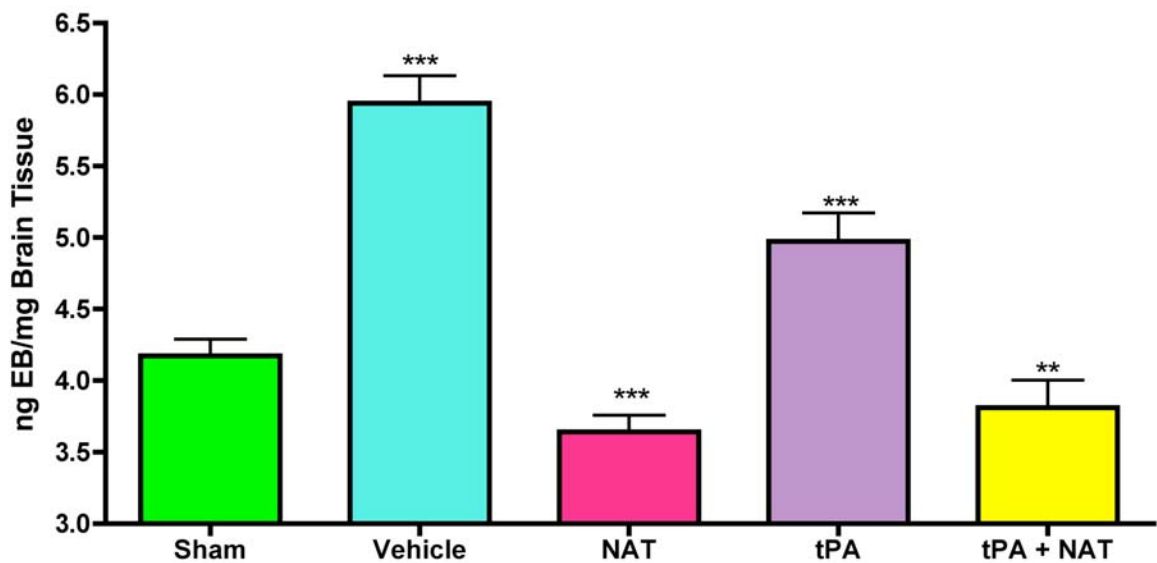
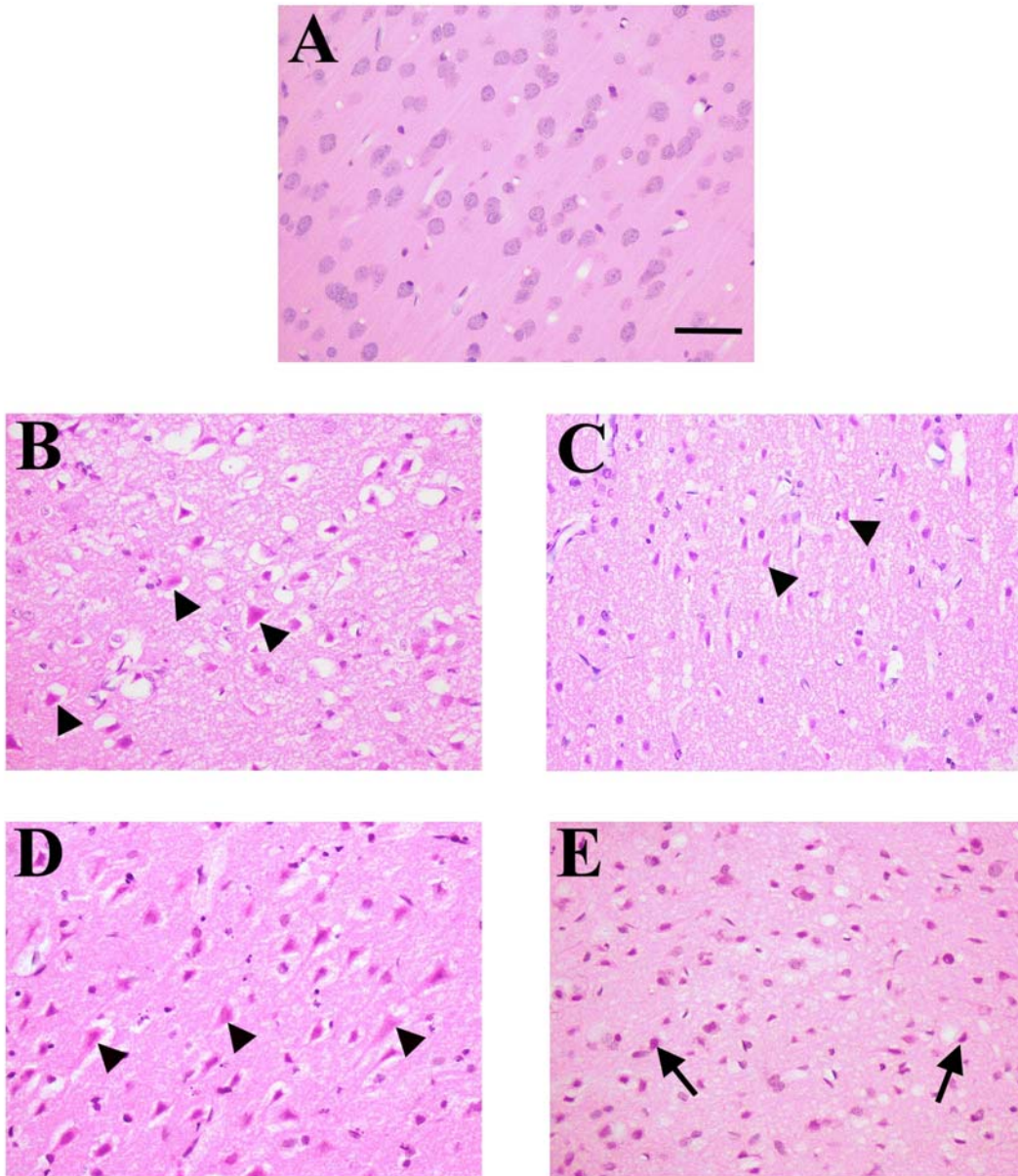**Figure 5.4 NAT/tPA combination therapy. EB extravasation at 24 h post-reperfusion.**

Following stroke, a significant increase ($p < 0.001$) in BBB permeability to EB was observed. Administration of NAT reduced EB extravasation to levels comparable to shams ($p > 0.05$). Administration of tPA significantly ($p < 0.001$) reduced EB extravasation as compared to vehicles, however the BBB permeability was still significantly greater than the NAT-treated group. The combination therapy of NAT and tPA significantly reduced EB extravasation to sham levels ($p > 0.05$). There was no significant ($p > 0.05$) difference between the EB extravasation in the NAT and combination therapy groups (**denotes $p < 0.01$; ***denotes $p < 0.001$; Sham n=7; Vehicle n=7; NAT n=5; tPA n=3; tPA+NAT n=5).

5.3.5 Effect of tPA on Histological Outcome

General Pathology: H&E

H&E staining was used to assess the effect of tPA treatment on the degree of tissue injury within the cortex and white matter at 24 h and 7 d post-reperfusion. No abnormalities were observed in the cortex of sham animals (Figure 5.5), indicating that sham surgery had no effect on cortical tissue architecture. At 24 h post-reperfusion, extensive vacuolation, cellular injury and cell loss was observed within the cortex. Treatment with NAT resulted in a reduction in the tissue vacuolation and the degree of cellular injury observed. Treatment with tPA had little effect on the degree of cell injury, although a modest reduction in tissue vacuolation was observed. However, the combination therapy of NAT/tPA resulted in a reduction in the cellular injury and tissue vacuolation observed within the cortex. By 7 d post-reperfusion profound reactive gliosis occupied the cortex (Figure 5.6). Treatment with NAT resulted in a modest reduction in the reactive gliosis, and a degree of tissue preservation was observed. A similar pattern was seen following treatment with tPA. However, the combination therapy of NAT/tPA resulted in the most profound preservation of cortical tissue with regions of normal parenchyma visible.
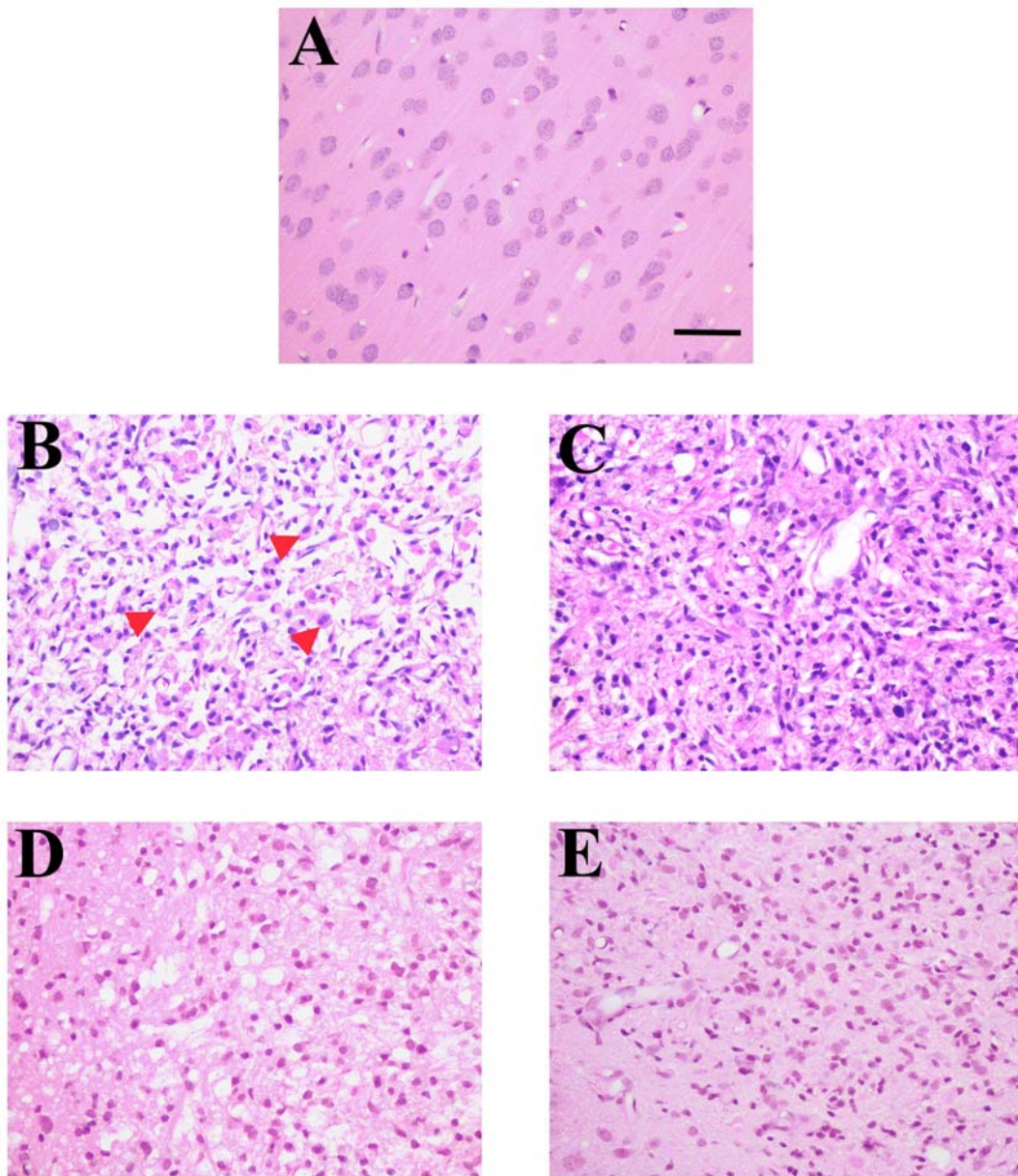
No abnormalities were observed in the white matter of sham animals (Figure 5.7), indicating that sham surgery had no effect on white matter tissue integrity. At 24 h post-reperfusion, extensive tissue injury was observed within the white matter with marked tissue vacuolation and cell loss evident. Treatment with NAT or tPA alone had little affect on the degree of injury observed within the white matter. However, combination of NAT/tPA resulted in a marked reduction in tissue vacuolation and cellular injury observed. By 7 d post-reperfusion, profound destruction of the white

Figure 5.5 NAT/tPA combination therapy. Cortex at 24 h post-reperfusion. H&E stained sections (Bar = 100 μm).

No abnormalities were observed in sham animals (A). At 24 h post-reperfusion (B) extensive vacuolation, cell injury (arrowheads) and cell loss was observed within the cortex. Treatment with NAT resulted in a reduction in the tissue vacuolation and the degree of cell injury observed (C). Treatment with tPA had little effect on the degree of cell injury (D). Combination of NAT and tPA (E) resulted in a reduction in the cell injury and vacuolation observed, although some DCC was still apparent (arrows).
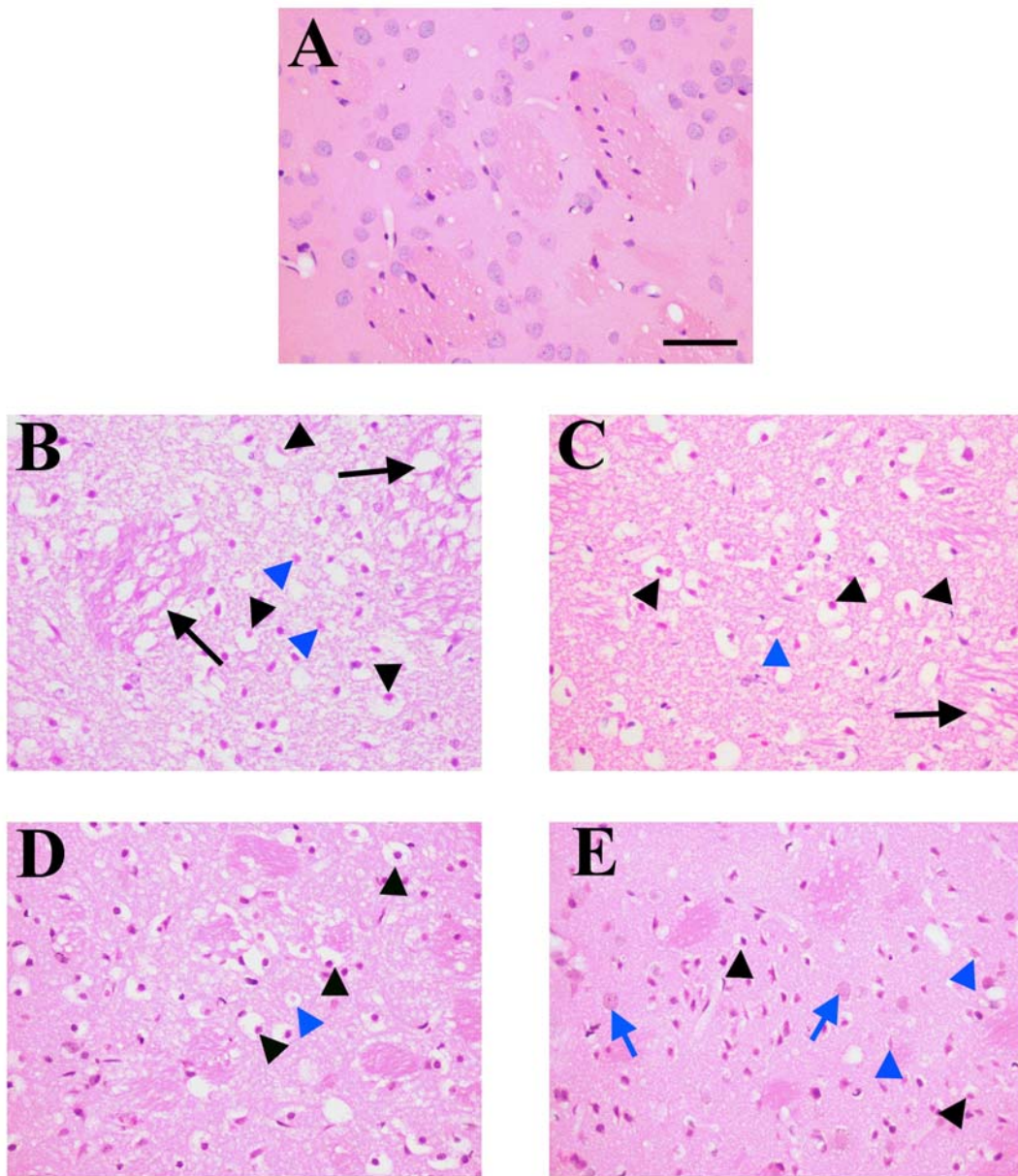
Figure 5.6 NAT/tPA combination therapy. Cortex at 7 d post-reperfusion. H&E stained sections (Bar = 100 μm).
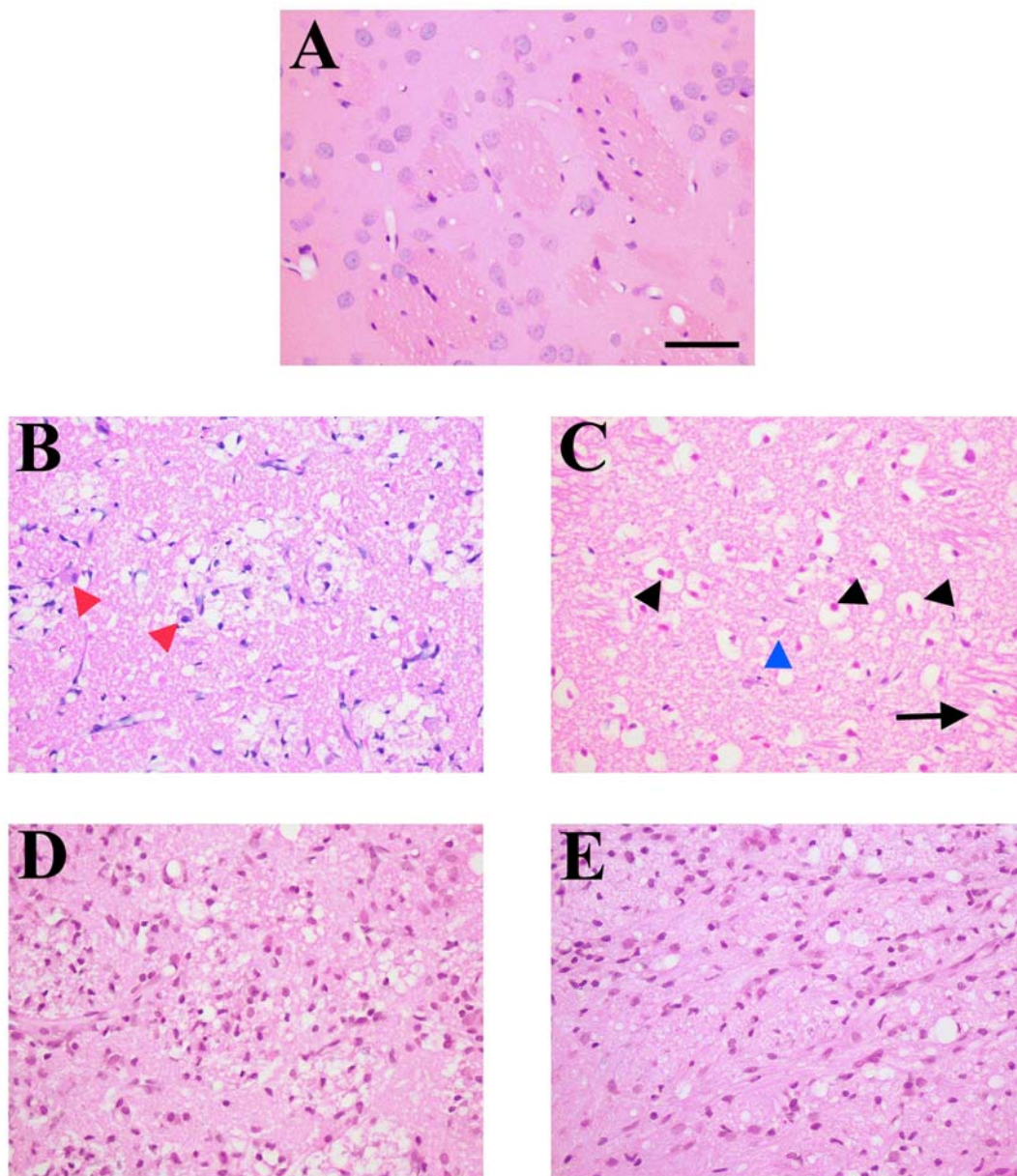
No abnormalities were observed in sham tissue (A). By 7 d post-reperfusion (B), profound reactive gliosis occupied the cortex, with an influx of macrophages also observed (red arrowheads). Treatment with NAT resulted in a modest reduction in the reactive gliosis and a degree of tissue preservation was observed. A similar pattern was seen following treatment with tPA (D). Combination of NAT and tPA resulted in a marked preservation of cortical tissue (E).

Figure 5.7 NAT/tPA combination therapy. White matter at 24 h post-reperfusion. H&E stained sections (Bar = 100 μm).

No abnormalities were observed in sham tissue (A). At 24 h post-reperfusion (B), extensive vacuolation of the white matter bundles (black arrows) and parenchyma (black arrowheads) and cell loss were observed within the white matter. Treatment with NAT (C) or tPA (D) had no affect on white matter injury. Combination of NAT and tPA (E) resulted in a reduction in tissue vacuolation and cell injury within the white matter, with some normal neurons observed (blue arrowheads).

Figure 5.8 NAT/tPA combination therapy. White matter at 7 d post-reperfusion. H&E stained sections (Bar = 100 μm).

No abnormalities were observed in sham tissue (A). By 7 d post-reperfusion (B), profound destruction of the white matter was observed, including vacuolation of the parenchyma (black arrowheads) and RCC (blue arrowheads), along with the influx of macrophages (red arrowheads). Treatment with NAT (C) or tPA (D) did not affect the degree of white matter injury observed. Combination of NAT and tPA resulted in a degree of tissue preservation.

matter was observed (Figure 5.8). As was the case at 24 h post-reperfusion, treatment with NAT or tPA had little affect on the degree of white matter injury observed but the combination of NAT/tPA afforded a degree of protection with regions tissue preservation observed at 7 d following stroke.

SP Response: SP Immunohistochemistry

Immunohistochemistry for SP was carried out to determine the effect of tPA treatment on the perivascular and cortical SP response at 24 h and 7 d post-reperfusion. Faint SP immunoreactivity was observed in the perivascular tissue of sham animals (Figure 5.9). At 24 h post-reperfusion, a profound increase in SP immunoreactivity around vessels was observed. Treatment with NAT reduced perivascular SP immunoreactivity. Treatment with tPA also resulted in some reduction in perivascular SP immunoreactivity, although this reduction was quite modest. Combination of NAT/tPA markedly reduced the SP immunoreactivity observed around blood vessels. By 7 d post-reperfusion, reactive gliosis occupied the cortex and little SP immunoreactivity was observed (Figure 5.10) and this response was largely unaffected by treatment with NAT, tPA or NAT/tPA.

Faint SP was observed within the cortex of sham animals (Figure 5.11). At 24 h post-reperfusion an increase in the SP immunoreactivity of cortical neurons was observed. NAT treatment reduced cortical SP immunoreactivity, while treatment with tPA did not affect cortical SP immunoreactivity. However, the combination of NAT/tPA reduced the SP immunoreactivity of cortical neurons to levels comparable to shams. By 7 d post-reperfusion extensive reactive gliosis occupied the cortex and
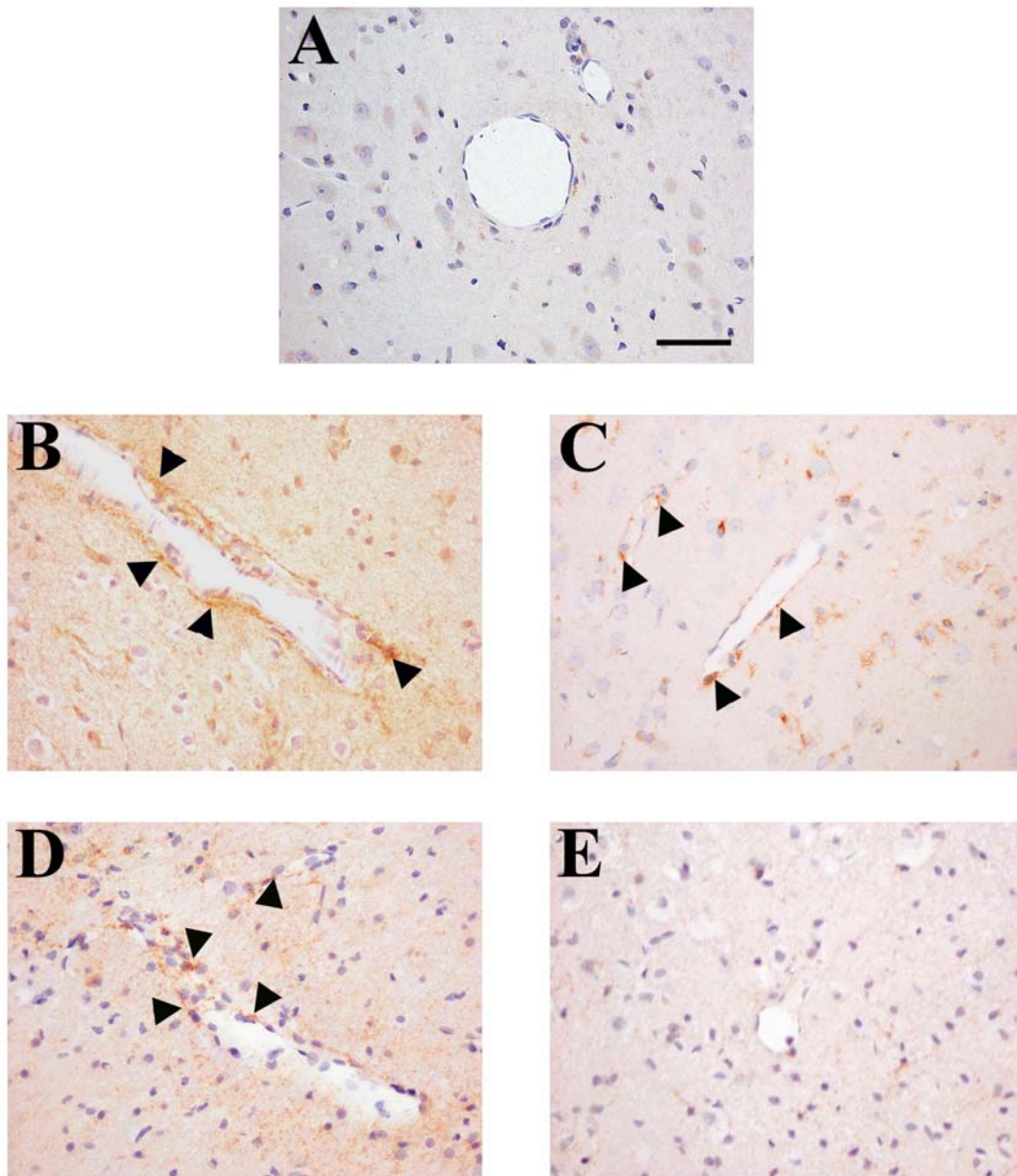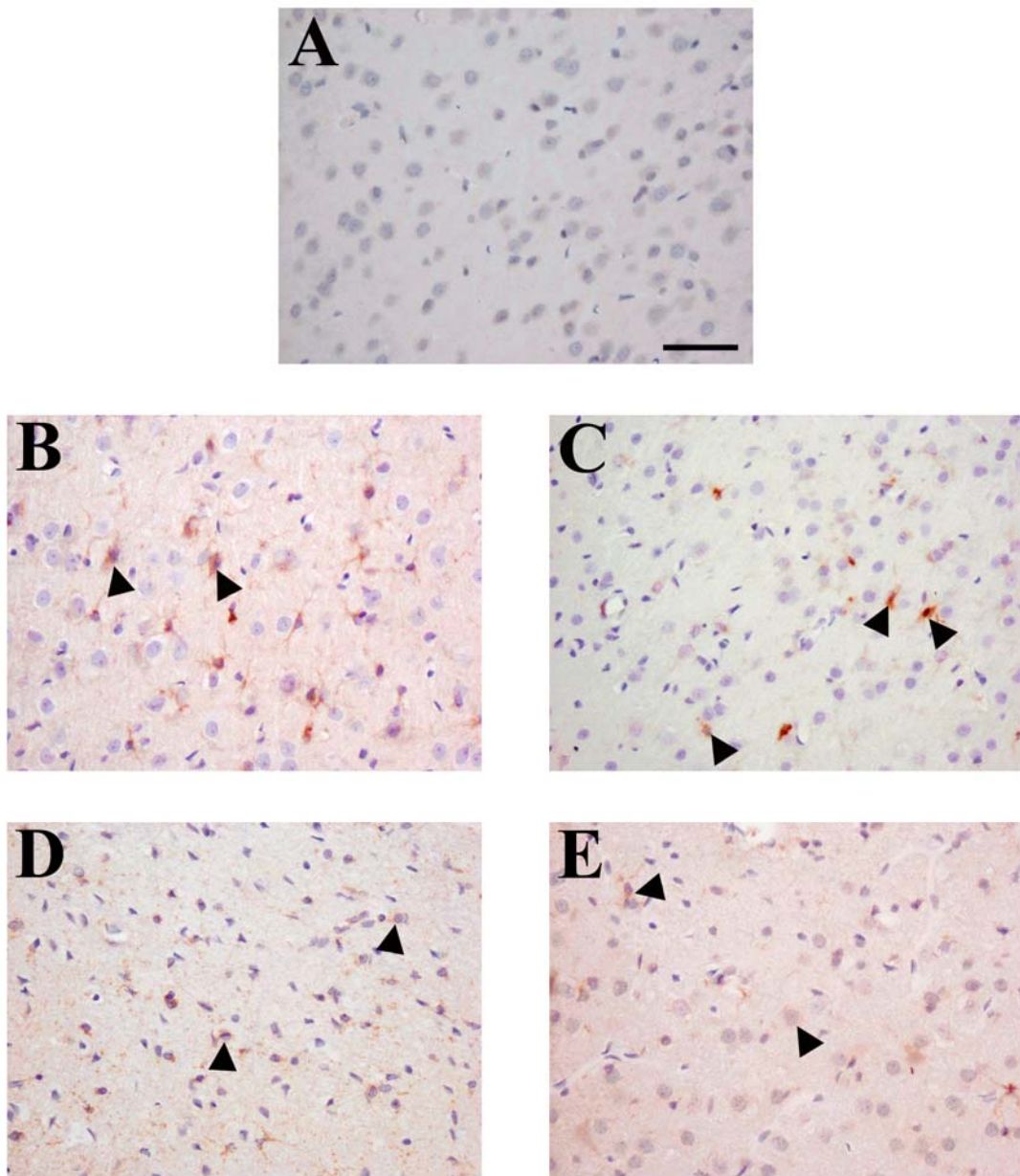
Figure 5.9 NAT/tPA combination therapy. SP Perivascular tissue at 24 h post-reperfusion. SP stained sections (Bar = 100 μm).

Faint perivascular SP immunoreactivity was observed in sham tissue (A). At 24 h post-reperfusion (B), a profound increase in SP immunoreactivity (arrowheads) around vessels was observed. NAT treatment (C) reduced perivascular SP immunoreactivity. tPA treatment (D) resulted in a modest reduction in perivascular SP immunoreactivity. Combination of NAT and tPA also reduced perivascular SP immunoreactivity.
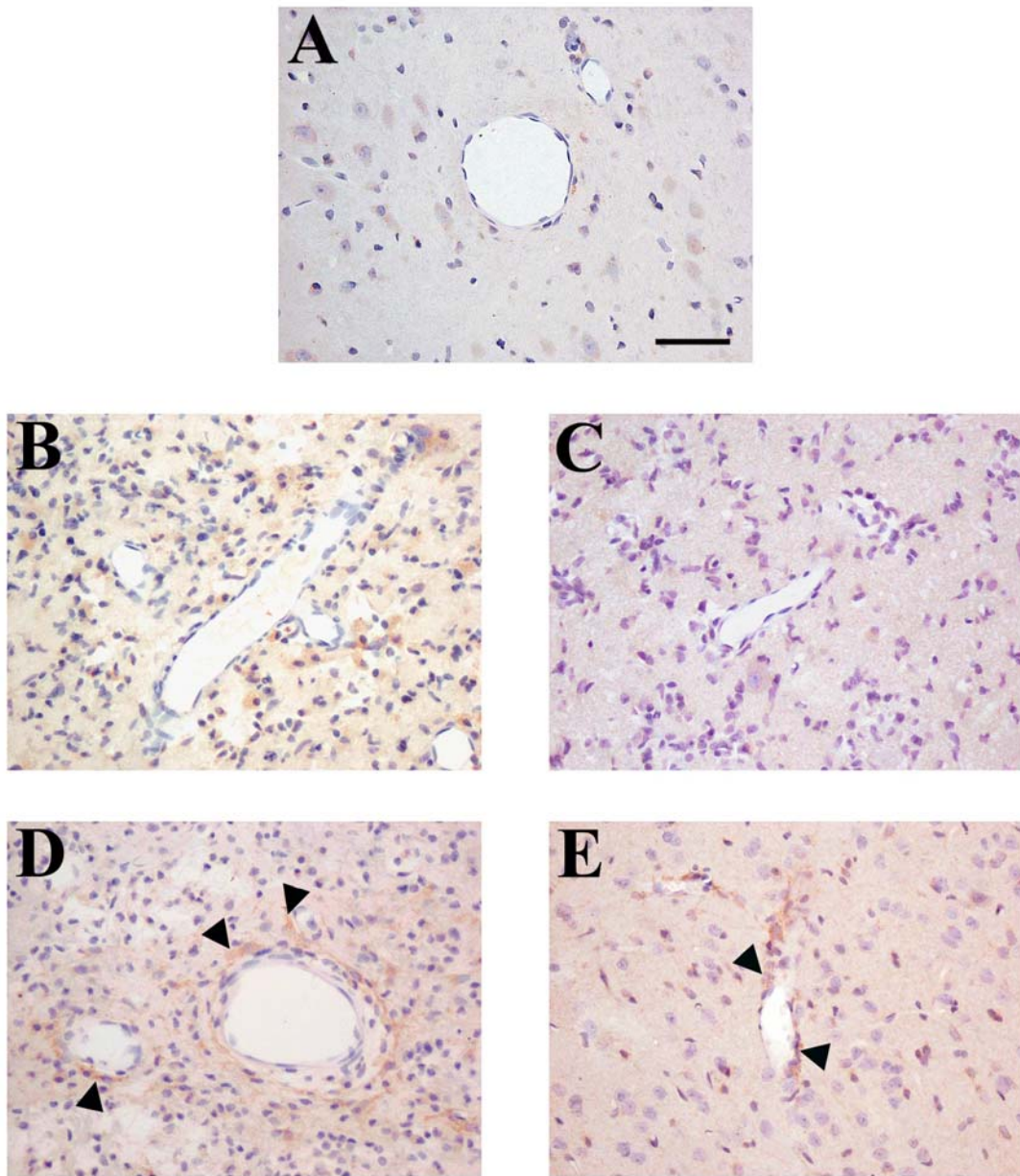
Figure 5.10 NAT/tPA combination therapy. Perivascular SP immunoreactivity at 7 d post-reperfusion. SP stained sections (Bar = 100 μm).
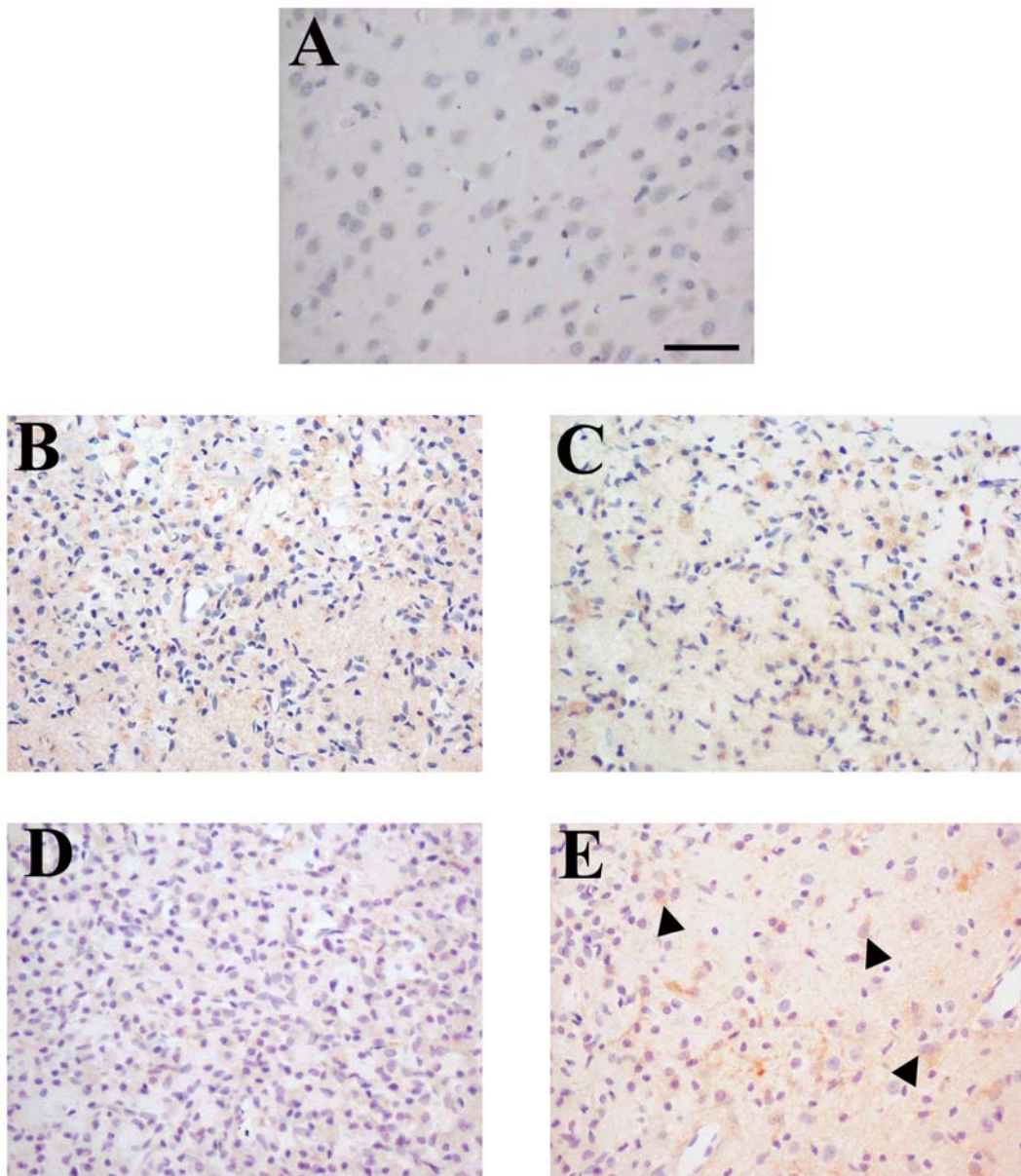
Faint SP immunoreactivity was seen in the perivascular tissue of sham animals (A). At 7 d post-stroke, reactive gliosis occupied the infarct, little perivascular SP immunoreactivity (arrowheads) was observed in vehicle (B), NAT (C), tPA (D) or NAT/tPA-treated animals (E). However a preservation of tissue was also observed within the NAT/tPA-treated group.

Figure 5.11 NAT/tPA combination therapy. Cortical SP Immunoreactivity at 24 h post-reperfusion. SP stained sections (Bar = 100 μm).

Faint SP was observed within sham tissue (A). At 24 h post-reperfusion (B) an increase in the SP immunoreactivity of cortical neurons (arrowheads) was observe. Treatment with NAT (C) reduced cortical SP immunoreactivity. tPA treatment did not affect cortical SP immunoreactivity. Combination of NAT and tPA (E) reduced the SP immunoreactivity of cortical neurons.

Figure 5.12 NAT/tPA combination therapy. Cortical SP immunoreactivity at 7 d post-reperfusion. SP stained sections (Bar = 100 μm).

Faint SP immunoreactivity was observed in sham tissue (A). At 7 d post-reperfusion (B), reactive gliosis occupied the cortex and little SP immunoreactivity (arrowheads) was observed. This was largely unaffected by NAT (C), tPA treatment. NAT/tPA treatment reduced the degree of cortical injury. Note the preserved neurons amongst the reactive gliosis (E).

little SP immunoreactivity was observed. This response was largely unaffected by administration of tPA, NAT or NAT/tPA. However, a reduced reactive gliosis reaction was observed in the NAT/tPA group, with preservation of neurons apparent.

Axonal Injury: APP Immunohistochemistry

Immunohistochemistry for APP was carried out to determine the effect of tPA treatment on the degree of axonal injury within the white matter and the response of cortical neurons to injury at 24 h and 7 d post-reperfusion. No axonal injury was observed in the white matter of sham animals (Figure 5.13), indicating that sham surgery had no effect on the integrity of the white matter tissue. At 24 h post-reperfusion, florid axonal injury was observed throughout the white matter. NAT treatment resulted in a modest reduction in axonal injury, although tPA treatment did not affect the degree of axonal injury observed. However, combination of NAT/tPA markedly reduced the axonal injury observed within the white matter, with few retraction balls evident. By 7 d post-reperfusion, florid axonal injury was still evident within the white matter, with large retraction balls observed (Figure 5.14). Treatment with NAT or tPA reduced the amount of axonal injury evident within the white matter at 7 d post-stroke, however combined NAT/tPA treatment produced the most profound decrease in axonal injury.
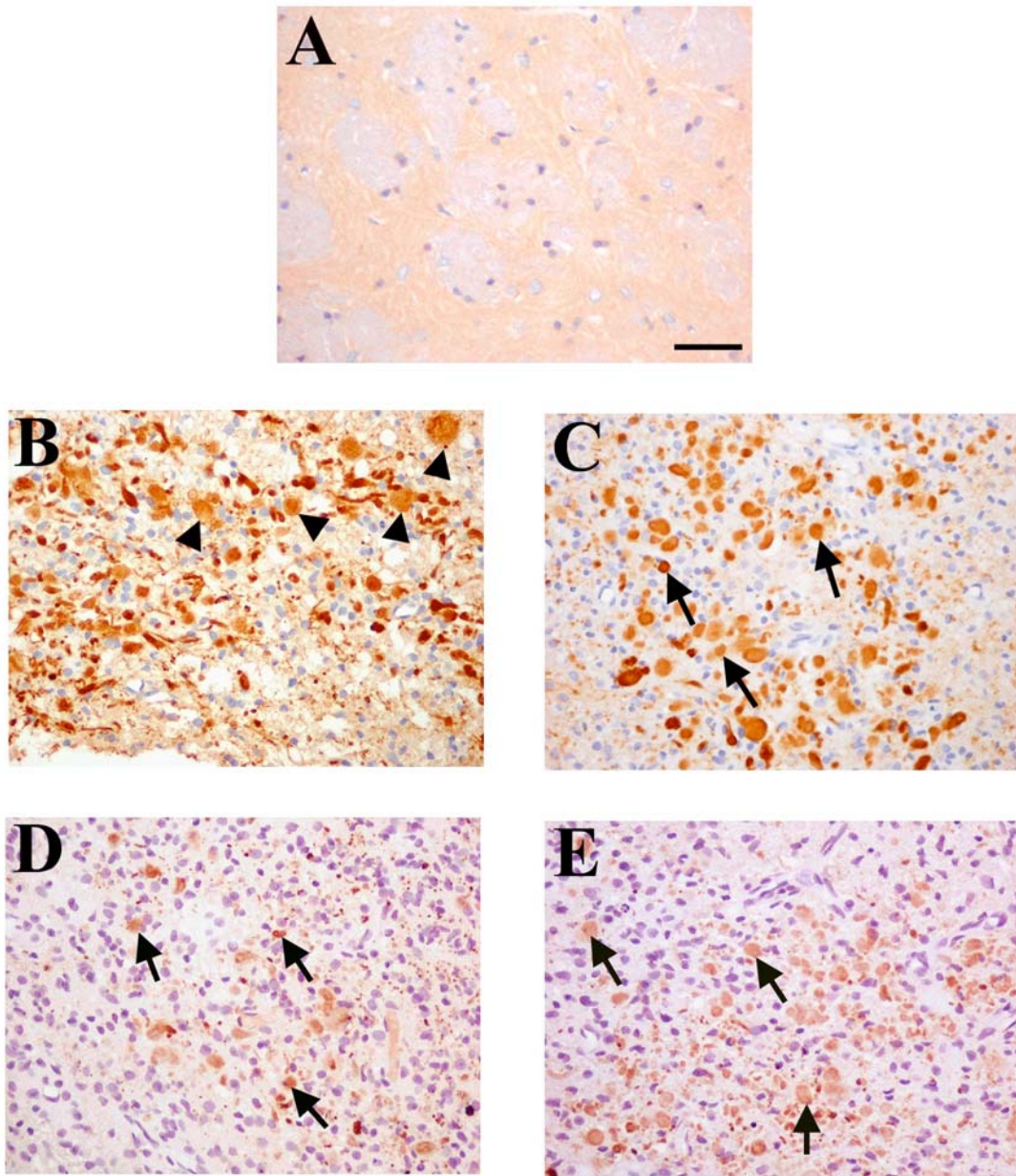

Faint APP immunoreactivity was observed within the cortex of sham animals (Figure 5.15), indicating that sham surgery had no effect on cortical APP immunoreactivity. At 24 h post-reperfusion, an increase in neuronal and
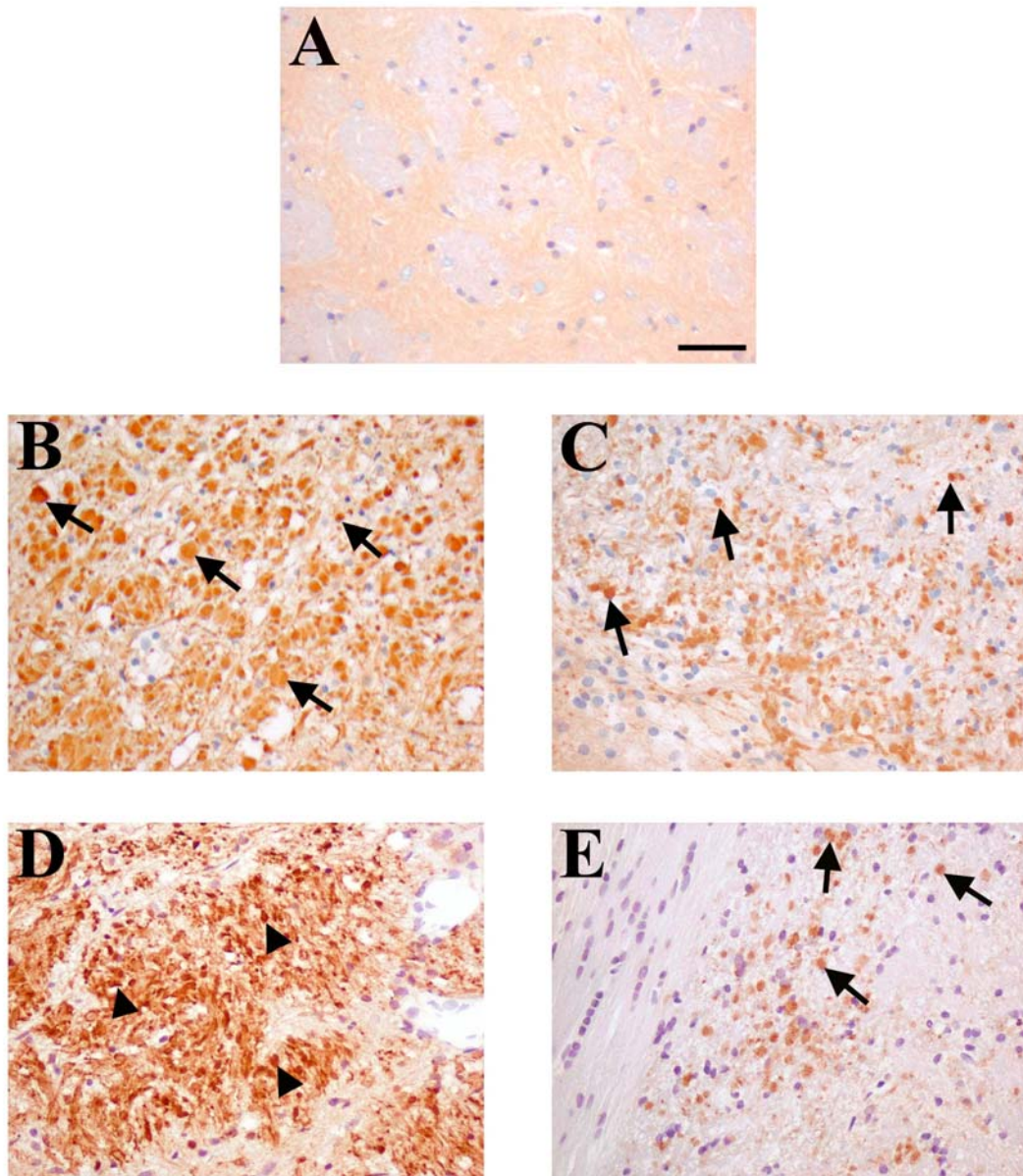
Figure 5.13 NAT/tPA combination therapy. Axonal Injury at 24 h post-reperfusion. APP stained sections (Bar = 100 µm).

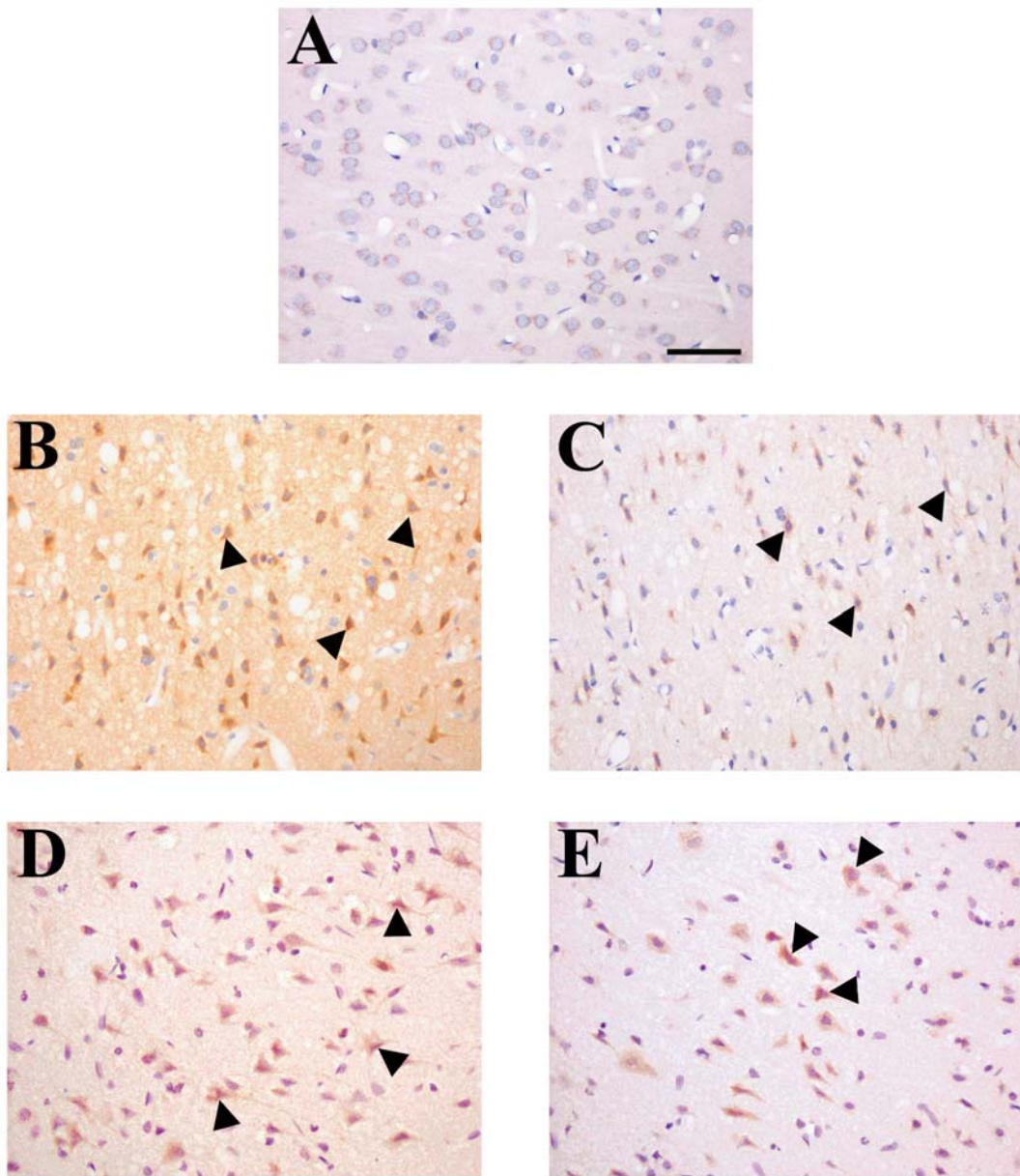No axonal injury was observed in sham tissue (A). At 24 h post-reperfusion (B), florid axonal injury (arrowheads) was seen throughout the white matter, observed as retraction balls. NAT treatment resulted in a modest reduction in axonal injury, while tPA treatment did not affect the degree of axonal injury (arrowheads) observed within the white matter. Combination of NAT and tPA markedly reduced axonal injury.

Figure 5.14 NAT/tPA combination therapy. Axonal Injury at 7 d post-reperfusion. APP stained sections (Bar = 100 μm).
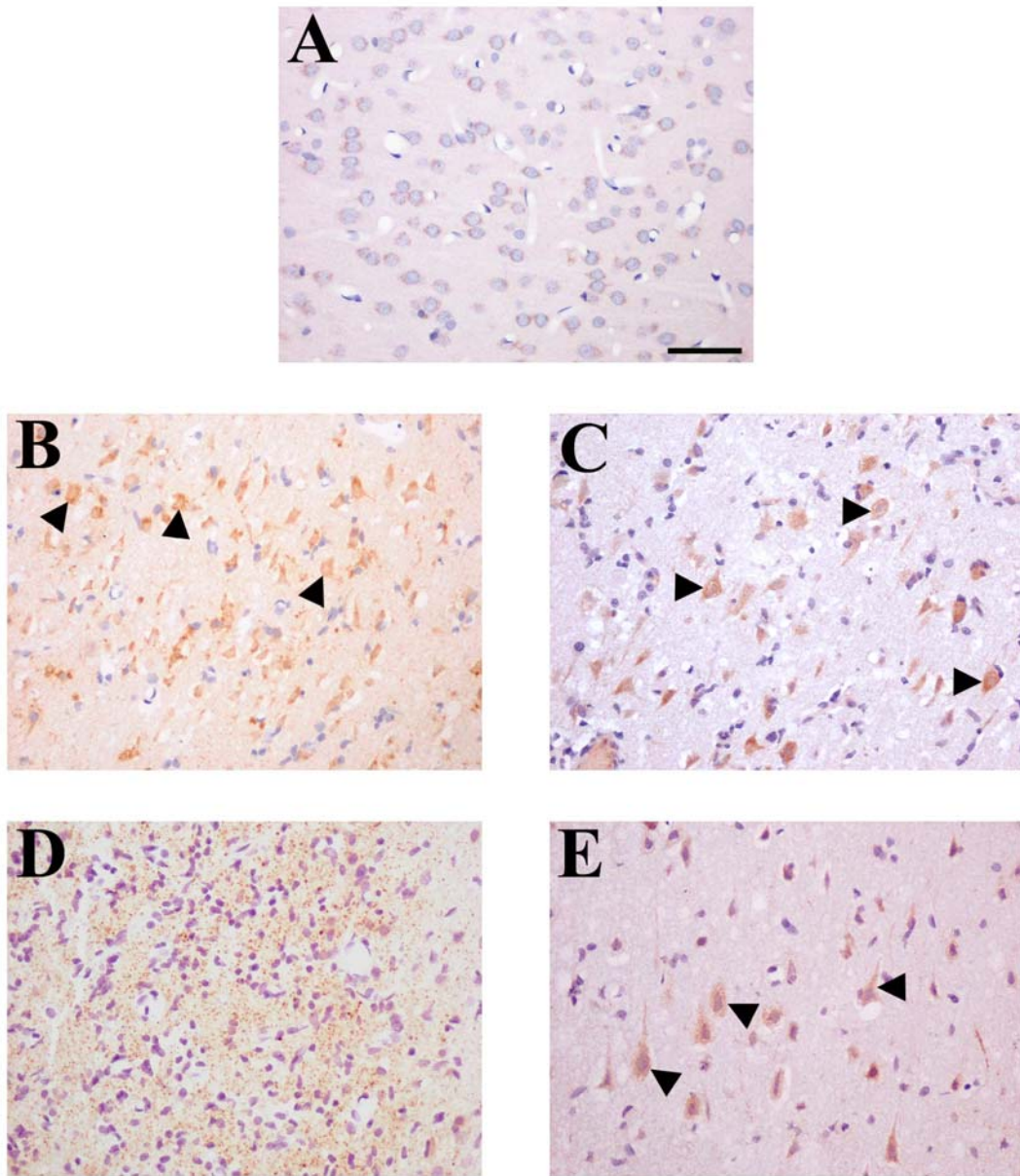
No axonal injury was observed in sham tissue (A). At 7 d post-reperfusion (B), florid axonal injury (arrowheads) was observed. NAT (C), tPA (D) and the combination therapy (E) all reduced the axonal injury (arrows) observed within the white matter at 7 d post-stroke.

Figure 5. 15 NAT/tPA combination therapy. Cortical APP Immunoreactivity at 24 h post-stroke. APP stained sections (Bar = 100 μm).

Faint APP immunoreactivity was observed in sham tissue (A). At 24 h post-reperfusion (B), an increase in neuronal (arrowheads) and parenchymal staining was observed within the cortex. Treatment with NAT (C), tPA (D) or the combination therapy (E) reduced parenchymal but not neuronal APP immunoreactivity.

Figure 5.16 NAT/tPA combination therapy. Cortical APP immunoreactivity at 7 d post-reperfusion. APP stained sections (Bar = 100 μm).

Faint APP immunoreactivity was observed in sham tissue (A). At 7 d post-reperfusion (B), increased neuronal (arrowheads) and parenchymal APP immunoreactivity was observed. Treatment with NAT (C) or the combination therapy (E) reduced parenchymal but not neuronal APP immunoreactivity. tPA treatment (D) did not affect cortical APP immunoreactivity.

parenchymal staining was observed within the cortex. Treatment with NAT or tPA, alone or in combination, reduced the degree of parenchymal but not neuronal APP immunoreactivity. By 7 d post-reperfusion, increased neuronal and parenchymal APP immunoreactivity was still evident within the cortex (Figure 5.16). Cortical APP immunoreactivity was unaffected by tPA treatment. Alternatively, treatment with NAT or the combination therapy (NAT/tPA) reduced parenchymal but not neuronal APP immunoreactivity.
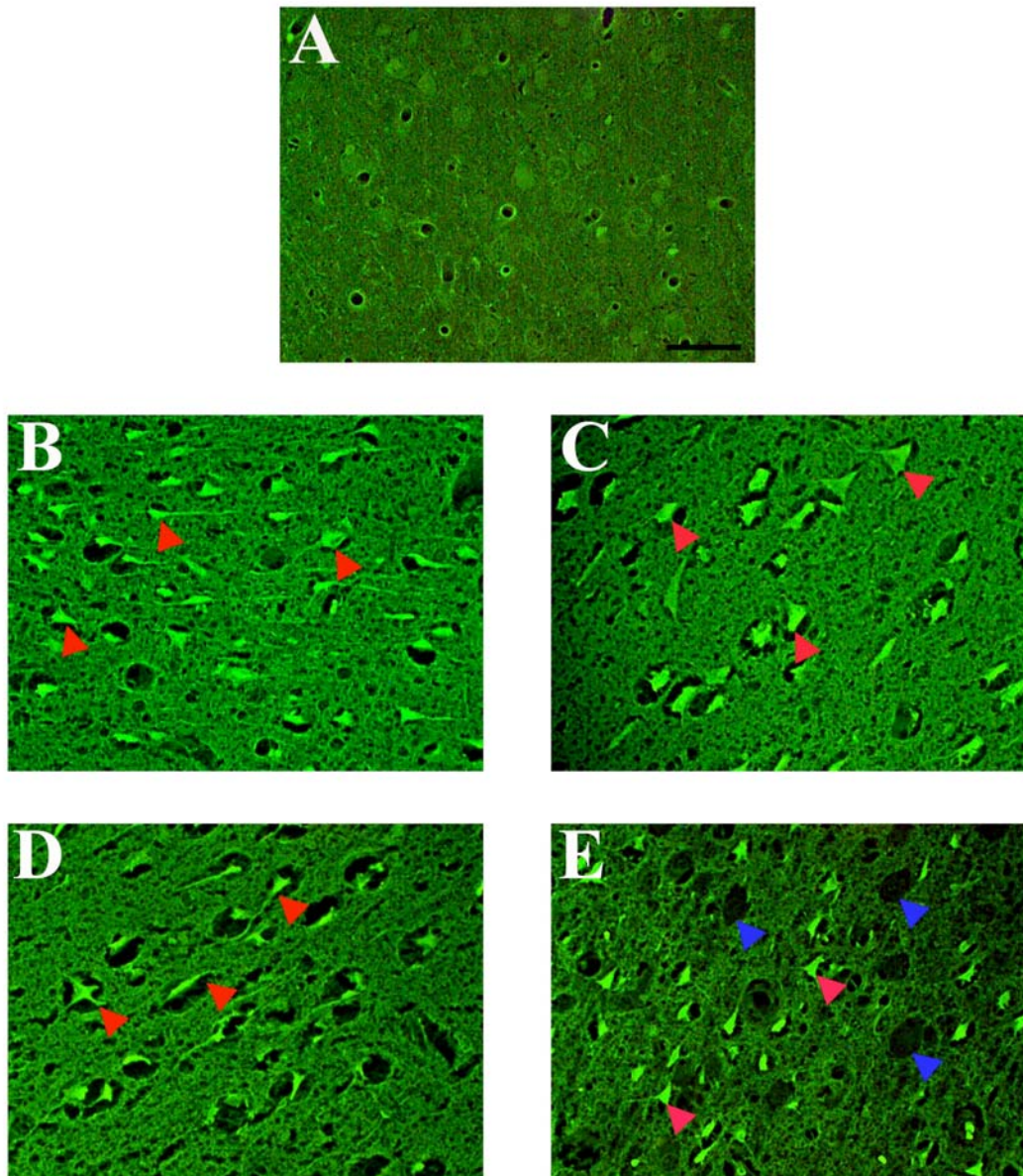
Degenerating Neurons: FJC

FJC staining was used to determine the effect of tPA treatment on the number of degenerating neurons within the cortex and white matter at 24 h and 7 d post-reperfusion. No degenerating neurons were observed in the cortex of sham animals (Figure 5.17), indicating that sham surgery did not affect neuronal survival. At 24 h post-reperfusion degenerating neurons were observed throughout the cortex and were associated with extensive tissue vacuolation. NAT or tPA treatment did not affect the amount of degenerating neurons. However, the combination therapy, NAT/tPA, resulted in some tissue preservation, with viable neurons observed alongside degenerating neurons within the cortex. By 7 d post-reperfusion, extensive reactive gliosis occupied the cortex and no degenerating neurons were observed (Figure 5.18). Treatment with NAT resulted in a modest reduction in the degree of reactive gliosis and some degenerating neurons were observed. A modest preservation in cortical architecture was observed following treatment with tPA or the combination therapy.

No degenerating neurons were observed within the white matter of sham animals (Figure 5.19), indicating that sham surgery did not affect neuronal survival. At 24 h post-reperfusion, degenerating neurons and marked vacuolation were observed throughout the white matter. Treatment with tPA did not affect the number of degenerating neurons within the white matter. Treatment with NAT or the combination therapy (NAT/tPA) resulted in a modest preservation in white matter architecture, although degenerating neurons were still observed. By 7 d post-reperfusion extensive reactive gliosis occupied the white matter (Figure 5.20). Treatment with tPA had no effect on the tissue architecture of the white matter however, NAT or NAT/tPA treatment resulted in a modest reduction in reactive gliosis such that a degree of tissue preservation and some degenerating neurons were observed.

Astrocyte response: GFAP

Light GFAP staining was observed within the perivascular tissue of sham animals (Figure 5.21), indicating that sham surgery did not affect astrocyte activation. An increase in the GFAP immunoreactivity around blood vessels was observed following 7 d of reperfusion, which was further exacerbated by treatment with NAT. The perivascular GFAP immunoreactivity observed in the tPA and combination therapy (NAT/tPA) groups was comparable to vehicle-treated animals. GFAP Immunohistochemistry was also used to determine the effect of tPA on the astrocytic response to injury within perivascular and penumbral tissue at 24 h and 7 d post-reperfusion. Light GFAP immunoreactivity of resting astrocytes was observed in sham tissue (Figure 5.22). Following 7 d of reperfusion, a marked increase in the number and activation of astrocytes was observed, which was further

Figure 5.17 NAT/tPA combination therapy. Degenerating neurons in the cortex at 24 h post-reperfusion. FJC stained sections (Bar = 100 μm).

No degenerating neurons were observed in sham tissue (A). At 24 h post-reperfusion (B) degenerating neurons (red arrowheads) were observed throughout the cortex and were associated with extensive tissue vacuolation. NAT (C) or tPA (D) treatment did not affect the amount of degenerating neurons. Combination of NAT and tPA (E) resulted in a degree of tissue preservation with viable neurons (blue arrowheads) observed alongside degenerating neurons.
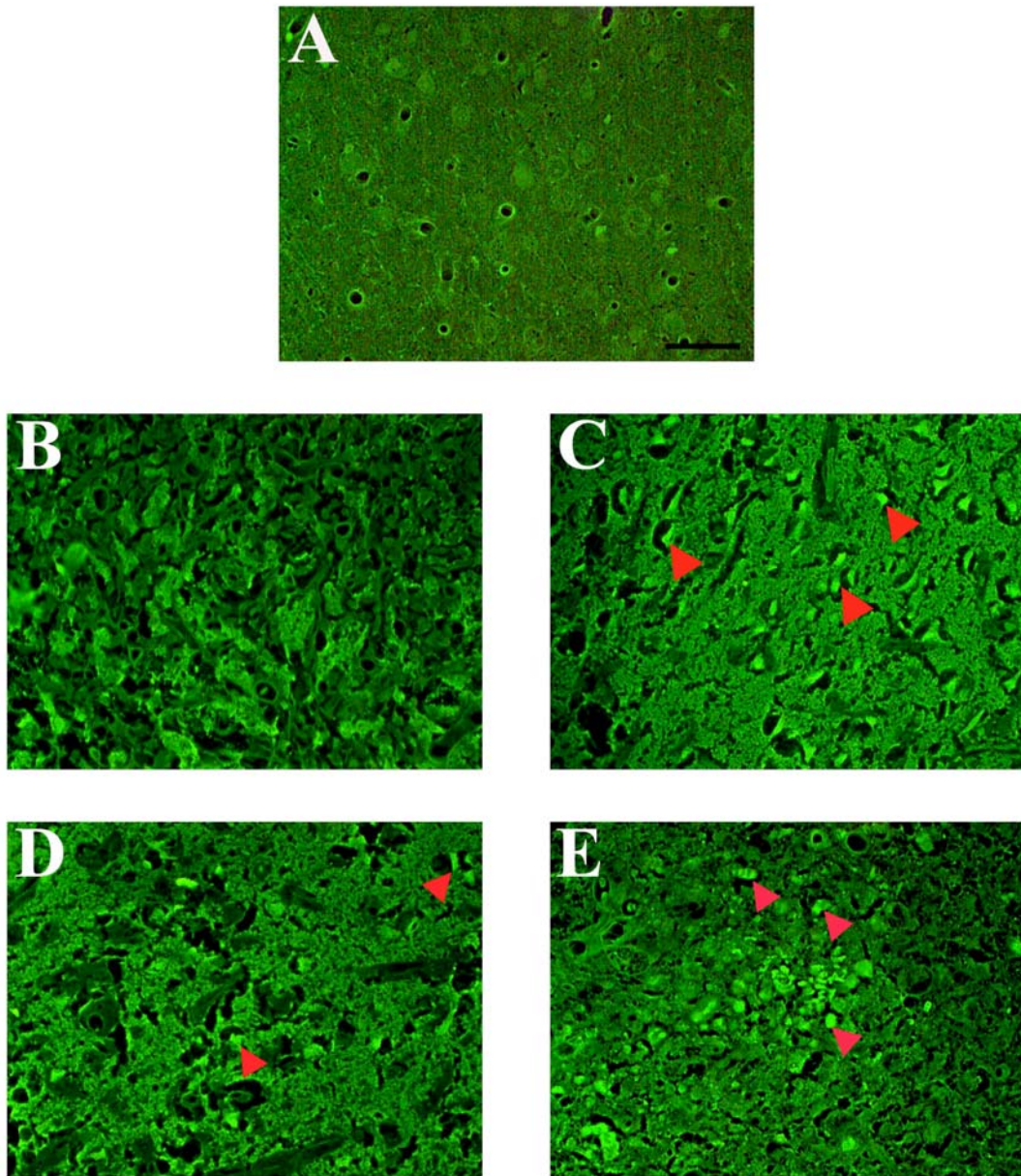
Figure 5.18 NAT/tPA combination therapy. Degenerating neurons in the cortex at 7 d post-reperfusion. FJC stained sections (Bar = 100 μm).

No degenerating neurons were observed in sham tissue (A). At 7 d post-reperfusion (B), extensive reactive gliosis occupied the cortex and no degenerating neurons were observed. Treatment with NAT (C) resulted in a modest reduction in the degree of reactive gliosis and some degenerating neurons (arrowheads) were observed. tPA (D) and combination therapy (E) resulted in modest tissue preservation.

Figure 5.19 NAT/tPA combination therapy. Degenerating neurons in the white matter at 24 h post-reperfusion. FJC stained sections (Bar = 100 μm).
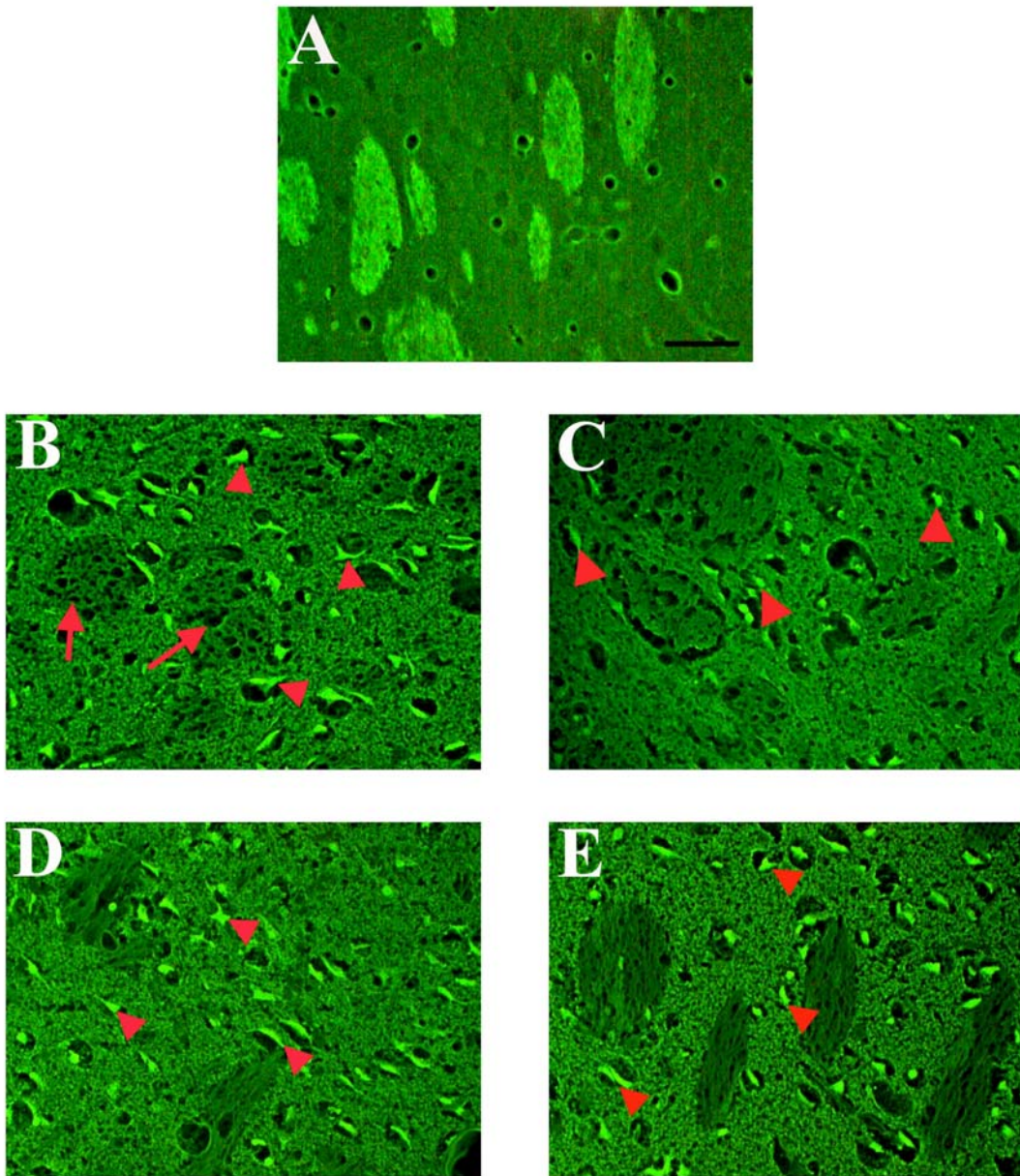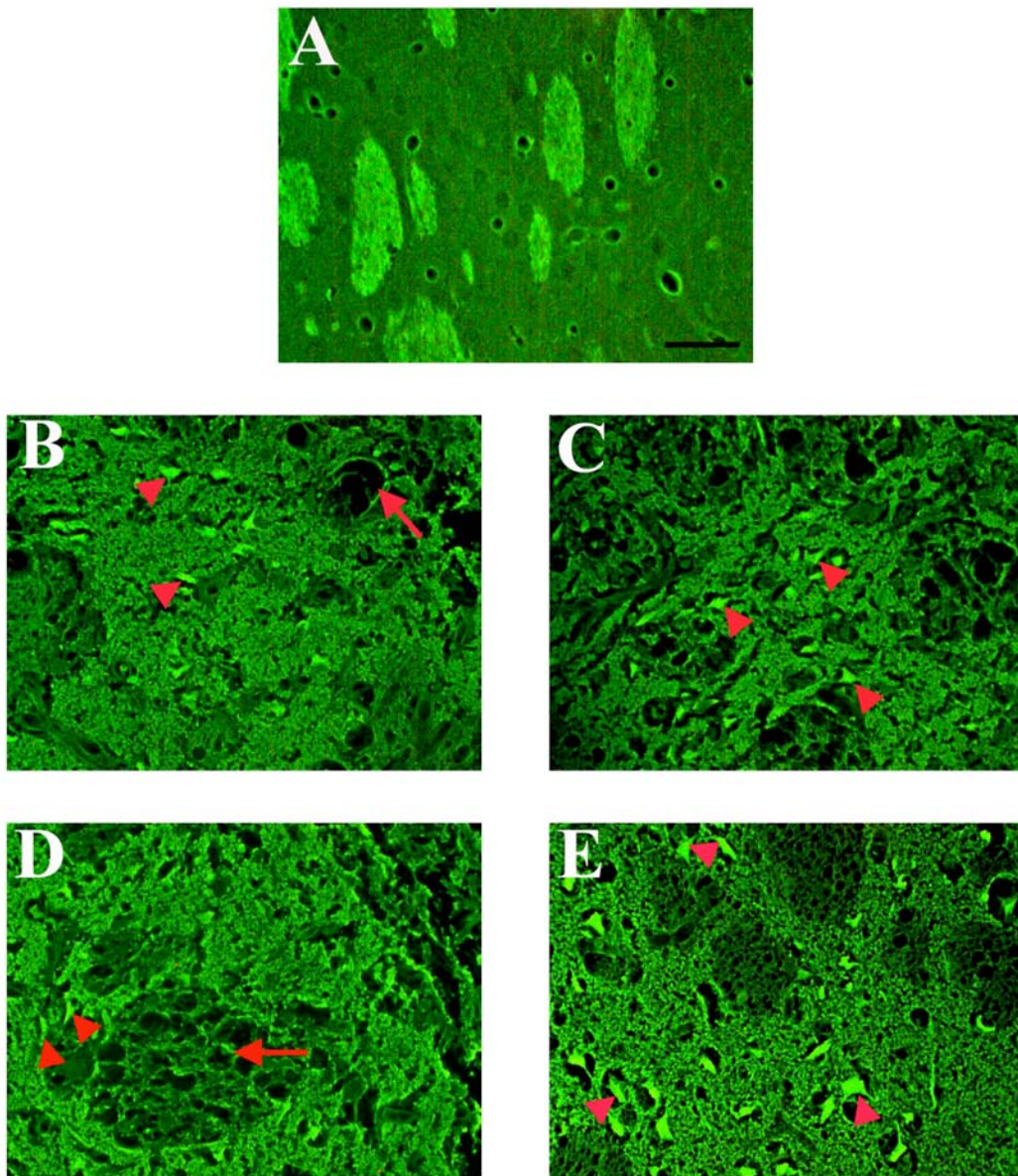
No degenerating neurons were observed in sham tissue (A). At 24 h post-reperfusion (B), degenerating neurons (arrowheads) and marked vacuolation were observed throughout the white matter, in addition to destruction of the white matter bundles (arrows). Treatment with tPA (D) did not affect the number of degenerating neurons within the white matter. Treatment with NAT (C) or the combination therapy (E) resulted in a modest preservation in white matter architecture, although degenerating neurons were still observed.

Figure 5.20 NAT/tPA combination therapy. Degenerating neurons within the white matter at 7 d post-reperfusion. FJC stained sections (Bar = 100 μm).

No degenerating neurons were observed in sham tissue (A). At 7 d following reperfusion (B), a marked loss of white matter architecture (arrows) was observed with reactive gliosis occupying the tissue, few degenerating neurons (arrowheads) were seen. NAT treatment resulted in a modest reduction in reactive gliosis and some degenerating neurons were observed (C). tPA treatment did not affect the degree of white matter injury (D). Treatment with NAT/tPA resulted in a marked improvement in white matter architecture, with vacuolation and some degenerating neurons still observed.

increased in the NAT-treated group. The astrocytic response to injury was comparable to vehicles in both the tPA and combination therapy (NAT/tPA) groups.

Macrophage/Activated Microglia Response: ED-1 Immunohistochemistry

Immunohistochemistry for ED-1 was used to determine the effect of tPA treatment on the extent of the macrophage/activated microglia response to injury at 24 h and 7 d post-reperfusion.   No macrophages/activated microglia were observed in association with blood vessels in sham tissue (Figure 5.23), indicating that sham surgery did not elicit an inflammatory response. Following 7 d of reperfusion, ED-1 positive were observed in close association with blood vessels. Treatment with NAT or tPA did not reduce the perivascular macrophage/activated microglia response, with tPA seeming to increase the number of macrophages relative to vehicle-treated animals. However, combination of NAT/tPA reduced the number of macrophages/activated microglia associated with vessels.

No macrophages/activated microglia were observed in the cortex of sham animals (Figure 5.24), confirming that sham surgery did not elicit an inflammatory response. A profound influx of macrophages/activated microglia was observed at 7 d post-reperfusion. Treatment with NAT, alone or in combination with tPA, resulted in a reduced macrophage/activated microglia response to injury. However, the influx of macrophages/activated microglia into the infarct was unaffected by treatment with tPA alone.
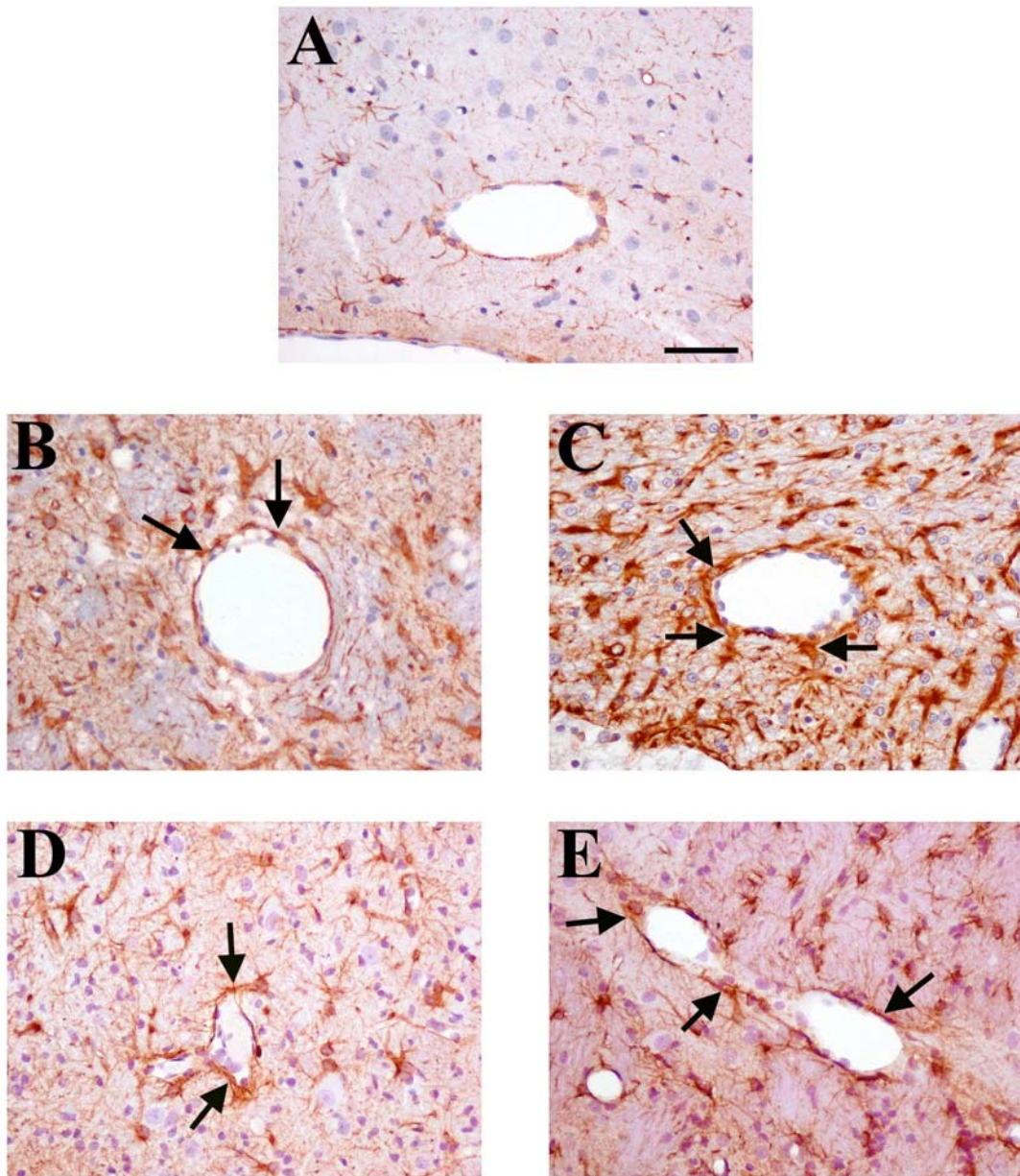
Figure 5.21 NAT/tPA combination therapy. Perivascular astrocytic response at 7 d post-reperfusion. GFAP stained sections (Bar = 100 μm).

Faint GFAP immunoreactivity was observed in sham tissue (A). At 7 d following reperfusion (B), an increase in GFAP immunoreactivity (arrows) was observed within perivascular tissue in all treatment groups (vehicle, NAT, tPA, NAT/tPA; B-E), however, it was most profound in the NAT-treated group (B).
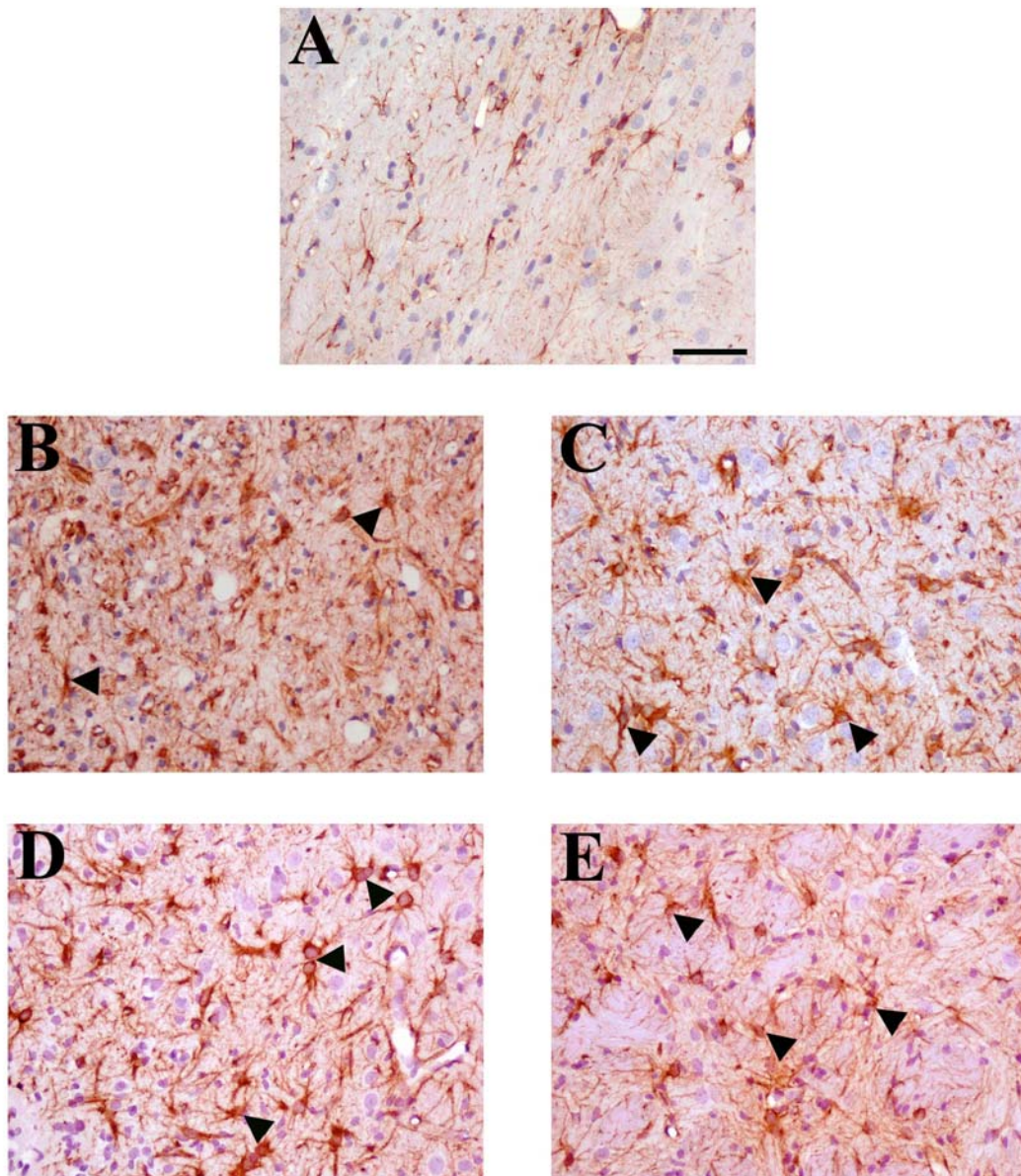
Figure 5.22 NAT/tPA combination therapy. Astrocytic response within the infarct border zone at 7 d post-reperfusion. GFAP stained sections (Bar = 100 μm).
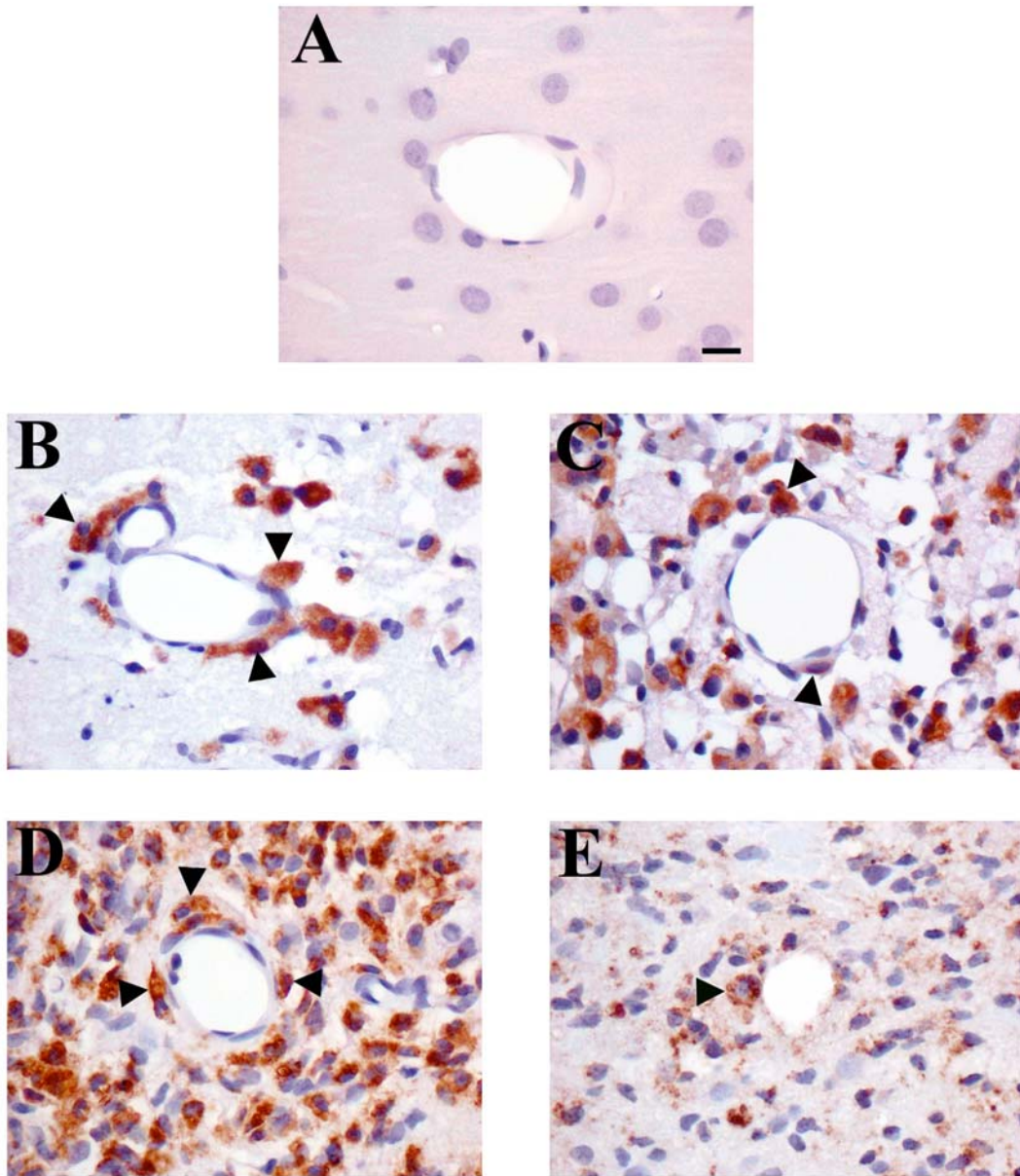
Faint GFAP immunoreactivity was observed in sham tissue (A). At 7 d following stroke (B), an increase in GFAP immunoreactivity (arrowheads) was observed in the infarct border zone. Treatment with NAT exacerbated this response to injury (C). Treatment with tPA (D) or NAT/tPA (E) produced comparable GFAP immunoreactivity within the infarct border zone.

Figure 5.23 NAT/tPA combination therapy. Perivascular Macrophage/ Activated Microglia response at 7 d post-reperfusion. ED-1 stained sections (Bar = 100 μm).

No macrophages were observed in association with blood vessels in sham tissue (A). At 7 d post-reperfusion (B), ED-1 positive macrophages (arrowheads) were observed in close association with blood vessels. This response was largely unaffected by treatment with NAT (C), tPA (D) or NAT/tPA (E).
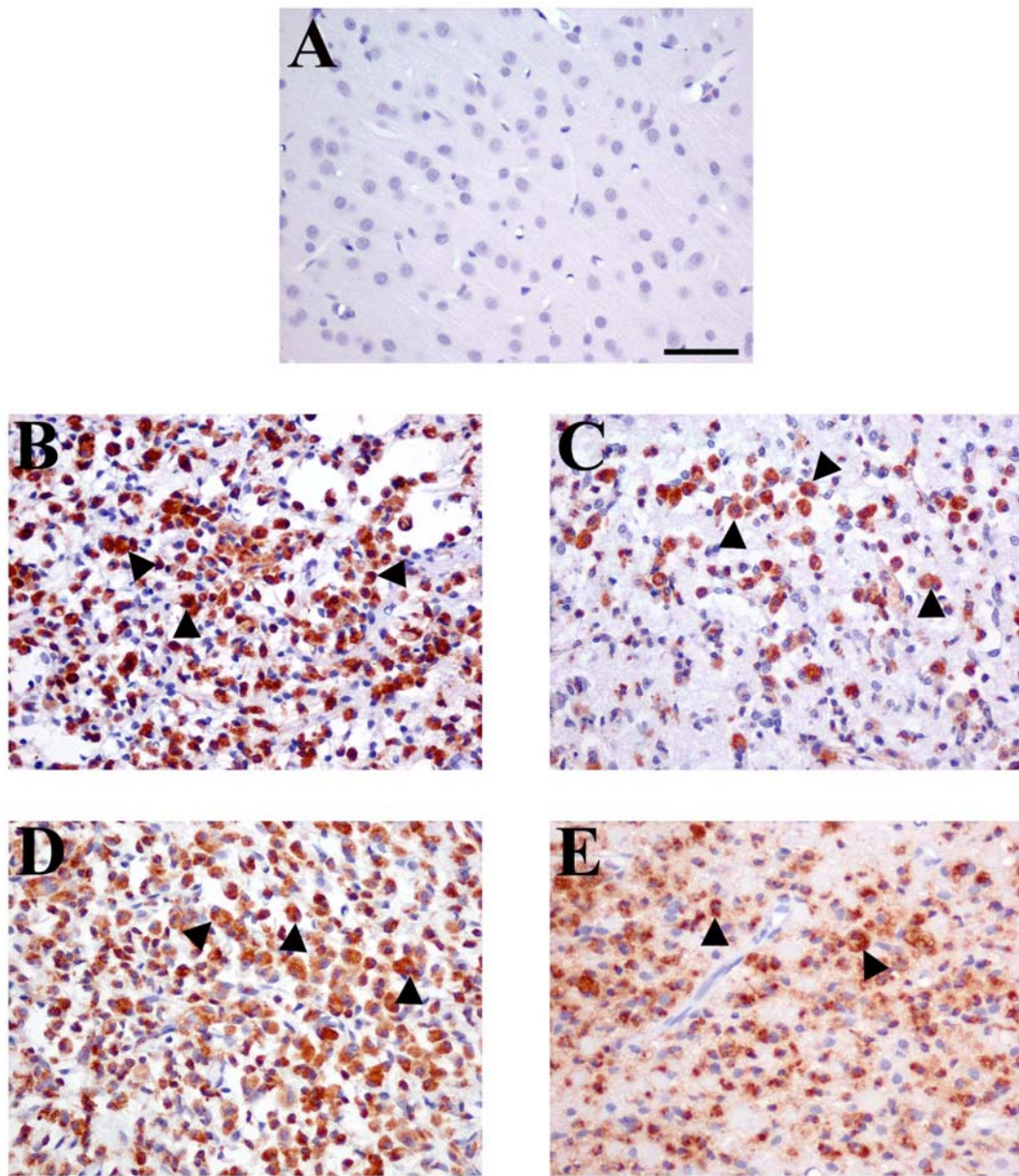
Figure 5.24 NAT/tPA combination therapy. Cortical Macrophage/Activated Microglia response within the infarct at 7 d post-reperfusion. ED-1 stained sections (Bar = 100 μm).

No macrohpage were observed within the cortex of sham animals (A). At 7 d post-reperfusion (B), a profound influx of ED-1 positive macrophages (arrowheads) was observed within the cortex. Treatment with NAT (C) or NAT/tPA (E) resulted in a reduction in the number of ED-1 positive cells within the cortex. However, tPA treatment had no effect on the number of ED-1 positive cells within the cortex (D).

5.3.4 Functional Outcome

Motor Function: Rotarod

Sham animals displayed no motor deficits on any of the assessment days, indicating that sham surgery had no effect on motor function. Following stroke, vehicle-treated animals showed profound motor deficits, scoring only 29 s on day 1 post-stroke. These deficits persisted for the 7 d assessment period. tPA-treated animals displayed a gradual improvement in their ability to walk on the rotarod over the assessment period, which was significantly better ($p<0.001$) than vehicle-treated animals. However, they did not reach normal functional levels at any time-point post-stroke, performing significantly worse than shams ($p<0.001$). NAT/tPA-treated animals were able to perform approximately 80 seconds on the rotarod on day 1 following stroke (Figure 5.25), in comparison to those animals treated with tPA alone that were only able to perform approximately 30 seconds. Over the course of the 7 d assessment period, animals in the NAT/tPA group were able to rapidly improve to reach normal functional levels by day 4 post-stroke. Statistical analysis revealed that NAT/tPA treated animals performed significantly better on the rotarod on all post-stroke assessment days ($p<0.001$) compared to vehicles and tPA-treated animals. The rotarod scores recorded for the NAT/tPA group were not significantly different from the NAT alone or sham groups (>0.05).

Sensory Function: Bilateral Asymmetry Test

Sham animals displayed no sensory deficits throughout the assessment period, indicating that sham surgery had no effect on sensory function. On day one following stroke the latency of vehicle animals to remove the tape was 114 s (Figure 5.26), in the tPA-treated group 85 s, the NAT-treated group 50 s, the NAT/tPA-
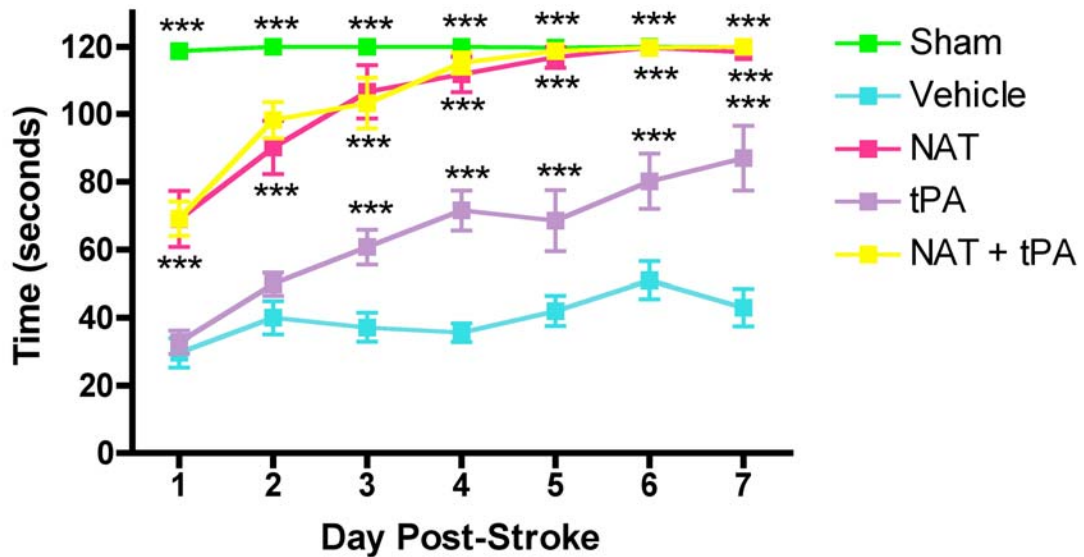
Figure 5.25 NAT/tPA combination therapy. Motor performance as assessed by the rotarod.

Sham animals had no motor deficits (green). Following stroke, vehicle animals (blue), showed marked motor deficits that persisted for the 7 d assessment period, performing significantly worse than shams (p<0.001). Treatment with tPA (purple) resulted in a gradual improvement in motor function over the 7 d assessment period and by day 3 they were significantly better than vehicle (p<0.001); however they did not reach normal functional levels. Treatment with NAT (pink) or NAT/tPA (yellow) resulted in a profound improvement in motor function as compared to vehicles, performing at levels not significantly different from shams by day 3 post-stroke (*** denotes p<0.001 compared to vehicle; Sham n=6, Vehicle n=6; NAT n=6; tPA n=9; NAT+tPA n=9).
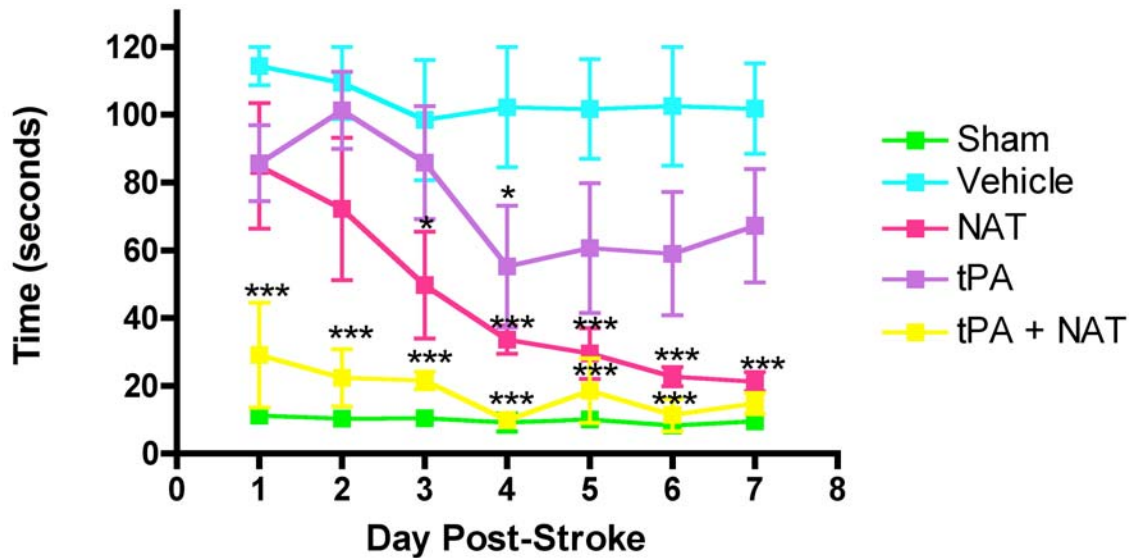
Figure 5.26 Figure 5.26 NAT/tPA combination therapy. Sensory neglect as assessed by the bilateral asymmetry test.

Sham animals had no sensory deficits (green). Vehicle animals (blue) demonstrated profound sensory deficits following stroke that persisted for the entire assessment period. Treatment with tPA (purple) resulted in a modest reduction in sensory deficits compared to vehicles. NAT-treated (pink) animals showed steady improvement, recording latencies not significantly different ($p>0.05$) than shams by day 5 post-stroke. NAT/tPA-treated (yellow) animals performed at levels comparable ($p>0.05$) to shams on all days post-stroke (* denotes $p<0.05$ versus vehicle; *** denotes $p<0.001$ versus vehicle) (Sham n=6, Vehicle n=6; NAT n=6; tPA n=9; NAT+tPA n=9).

treated group 29 s and the sham group 11 s. Vehicle animals showed profound sensory deficits which persisted for the 7 d testing period, recording latencies significantly higher ($p<0.001$) than shams on all days post-stroke. Treatment with NAT resulted in a gradual decline in sensory deficits, such that by day 3 post-stroke latencies were not significantly ($p>0.05$) different from shams. The tPA treatment group showed a gradual improvement in sensory performance however this was only significantly better than vehicles on day 4 ($p<0.05$) post-stroke. The tPA group performed significantly ($0.05<p<0.001$) worse than shams on all days post-stroke. Treatment with NAT/tPA resulted in a profound reduction in sensory deficits. This group performed significantly better ($p<0.001$) than vehicles on all days post-stroke, recording latencies comparable ($p>0.05$) to shams. The combined therapy group (NAT/tPA) were the only group to record latencies not significantly different from sham animals ($p>0.05$) on all post-stroke assessment days.

Spontaneous Exploratory Behaviour: Open Field

Sham animals showed normal activity levels in the open field, indicating that sham surgery had no effect on spontaneous exploratory behaviour. The gradual decline in open field activity over time in the sham group is likely to reflect habituation to the task, which has been well documented in uninjured animals (McIlwain et al., 2001; Paylor et al., 2006; Stohr et al., 1998). Following stroke, vehicle-treated animals displayed a significantly lower ($p<0.05$) activity level compared to shams on day one post-stroke (Figure 5.27) and their activity level remained reduced for the whole assessment period. There was a trend towards decreasing activity over time, however this was not significant. NAT-treated animals showed a significantly ($p<0.05$) higher activity level compared to vehicle animals on day 1 post-stroke

only, and a trend towards improved open field activity for the remainder of the 7 d assessment period. The activity levels of the tPA group were significantly lower (p<0.05) than shams on day 1 post-stroke and in fact, comparable to vehicle-treated animals over the first half of the assessment period, before improving relative to vehicles. There was no significant (p>0.05) differences in the activity levels of the NAT/tPA group as compared to vehicle or shams, on any day post-stroke, with better scores apparent compared to both the tPA alone and vehicle groups. No significant differences were observed beyond day 1 post-stroke between any of the groups, and this is likely to reflect the large variability within each group.

Neurological Function: Modified Neuroseverity Score

Sham animals recorded no neurological deficits during the assessment period (Figure 5.28), confirming that sham surgery did not affect neurological function. Vehicle-treated animals had a significantly (p<0.001) greater neurological deficit compared to sham animals, with a ranking of 7 (moderate injury) on day 1 post-stroke. This moderate deficit persisted for the entire assessment period. On day 1 post-stroke, NAT-treated animals had a neuroscore value of 3 (mild injury), and the neurological deficit of these animals decreased over the assessment period. By day 5, there was no observable deficit and the injury ranking was not significantly (p>0.05) different to shams. tPA-treated animals had a neuroscore of 5.5 (moderate injury) on day one following stroke, and the neurological deficit of this group decreased to a ranking of mild injury by day 7 post-stroke. However, this improvement was not significantly (p>0.05) better than vehicles but was still significantly (p<0.01) worse than shams. On day 1 post-stroke, the NAT/tPA-treated group had a ranking of 2 (mild injury), demonstrating significantly improved

(p<0.01) neurological function compared to vehicles. A rapid improvement in neurological function was observed, with an injury ranking comparable (p>0.05) to shams on all assessment days. The combination therapy of NAT/tPA was clearly the most effective therapy for reducing neurological deficits following stroke.

Hemiparesis: Angleboard

No hemiparesis was observed in sham animals (Figure 5.29), indicating that sham surgery had no effect on muscle strength and balance. Following stroke, vehicle-treated animals demonstrated profound hemiparesis compared to shams (0.001<p<0.05), which did not improve over the 7 d assessment period. NAT treatment significantly reduced hemiparesis (0.001<p<0.05) compared to vehicles on days 1-2, and 4-6 post-stroke. Treatment with tPA also reduced the degree of hemiparesis, with animals in this group scoring significantly better (0.05<p<0.01) on the angleboard than vehicles on days 1-2 and 4-7 post-stroke. NAT/tPA treatment ameliorated hemiparesis (0.001<p<0.01) as compared to vehicles, on all assessment days post-stroke. All the drug treatments were effective in reducing hemiparesis following stroke and there was no significant difference between any of these treatment groups.

In summary, combined administration of NAT/tPA following stroke was a significantly better treatment strategy than tPA alone, as animals in the NAT/tPA treatment group returned to normal functional levels on all of the assessment tasks. In some instances the combined therapy was also more effective than NAT alone, suggesting a synergistic action in the combination treatment.
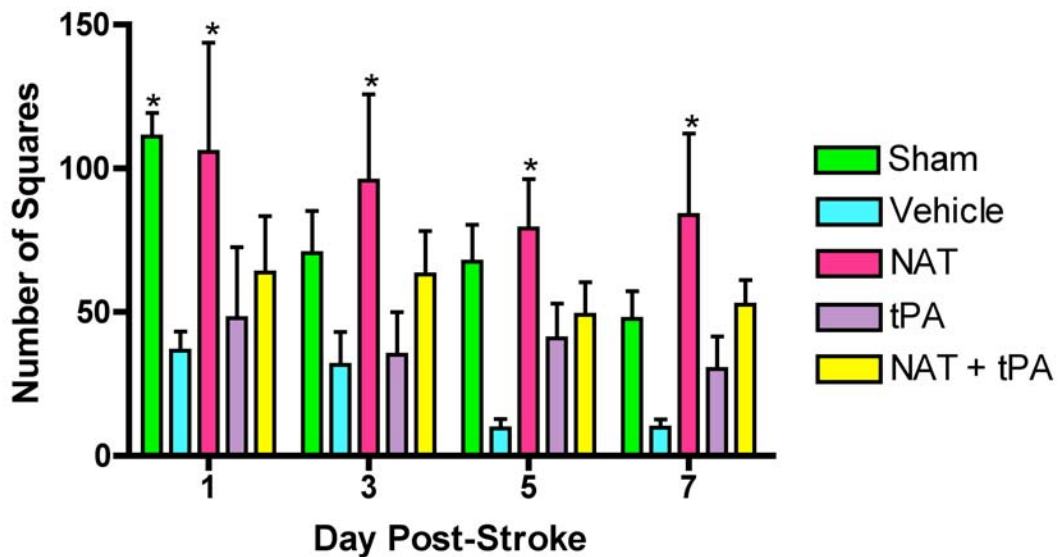
Figure 5.27 NAT/tPA combination therapy. Post-stroke stress and anxiety as assessed by the open field.

Sham animals (green) showed normal activity levels in the open field. Following stroke, vehicle animals (blue) demonstrated a significant decline ($p < 0.05$) in open field activity compared to shams that persisted for the 7 d assessment period. NAT treatment (pink) significantly improved ($p < 0.05$) activity levels, such that they were not significantly ($p > 0.05$) different from shams on any day post-stroke. There was a trend towards increased activity levels in the tPA (purple) and NAT-tPA (yellow) groups compared to vehicles (* denotes $p < 0.05$ versus vehicle) (Sham n=6, Vehicle n=6; NAT n=6; tPA n=9; NAT+tPA n=9).
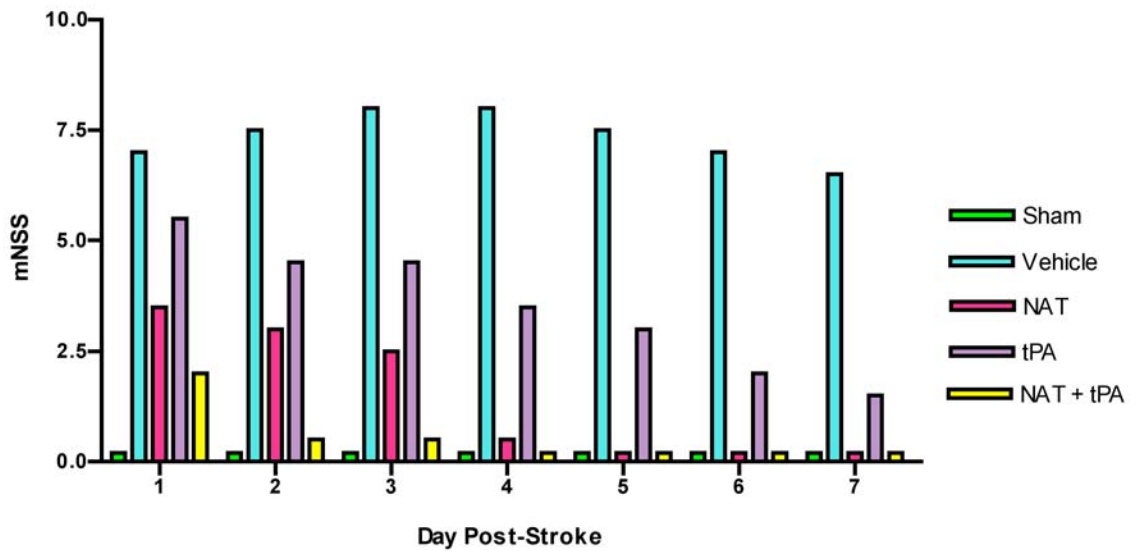
Figure 5.28 NAT/tPA combination therapy. Post-stroke neurological function as assessed by the mNSS.

Sham animals (green) had no neurological deficits. Following stroke, vehicle animals (blue) showed profound ($p<0.001$) neurological deficits that persisted for the 7 d assessment period. NAT/tPA (yellow) ($p<0.01$) and NAT treatment (pink) reduced neurological deficits compared to vehicles, and no recordable deficits were observed by days 4 and 5 respectively. tPA treatment (purple) also reduced neurological deficits, however mild deficits were still observed by day 7 post-stroke (Sham n=6, Vehicle n=6; NAT n=6; tPA n=9; NAT+tPA n=9).
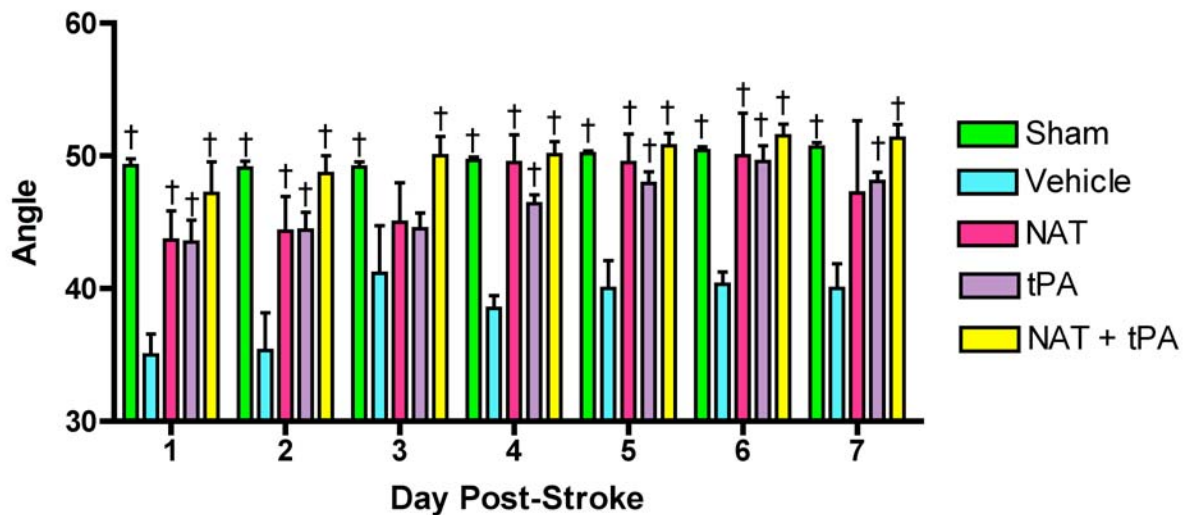
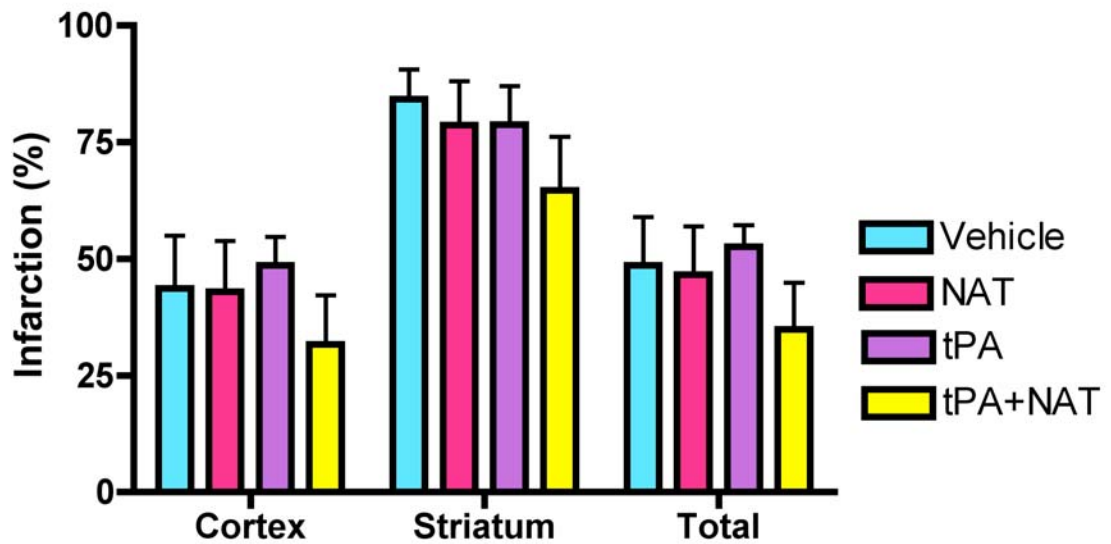**Figure 5.29 NAT/tPA combination therapy. Post-stroke hemiparesis as assessed by the angleboard.**

Sham animals (green) showed no hemiparesis. Following stroke, vehicle animals (blue) showed significant hemiparesis. Treatment with NAT (pink), tPA (purple) or NAT/tPA (yellow) reduced the degree of hemiparesis observed († denotes $0.001 < p < 0.05$ versus vehicle) (Sham n=6, Vehicle n=6; NAT n=6; tPA n=9; NAT+tPA n=9).

5.3.6 Effect of tPA on infarct volume

MCAO resulted in infarction of approximately 43% of the cortex and 84% of the striatum, equating to a total infarct of 48%. NAT administration alone had no effect (p>0.05) on the degree of infarction with 43% cortical and 78% striatal infarction observed, equating to a total of 46%. Similarly, administration of tPA at the onset of reperfusion had no effect (p>0.05) on the degree of infarction (Figure 5.30). However, the combined therapy of tPA and NAT showed a trend (p>0.05) towards reducing the degree of infarction, with 31% cortical and 65% striatal infarction, equating to a total of 35%. A reduction in infarct volume following administration of an NK_1 receptor antagonist (Yu et al., 1997) or exogenous tPA (Toomey et al., 2002) has previously been reported in experimental stroke, although a lack of reduction in infarct volume despite functional improvement has also been reported in a number of studies (Grotta et al., 1988; Grotta et al., 1990; Aronowski et al., 1994; Aronowski et al., 1996; van der Staay et al., 1996). In our studies, improvement in functional outcome was not associated with a reduction in infarct volume.

Figure 5.30 NAT/tPA combination therapy. Infarct volume at 24 h post-reperfusion, as assessed by TTC staining.

There was no significant difference between the degree of infarction in any of the treatment groups, with respect to the cortex, striatum and total area of infarction. However, there was a trend towards reduced cortical and striatal and total infarction in the NAT/tPA group (Vehicle n=7; NAT n=9; tPA n=5; NAT+tPA n=4).

5.4 Discussion

The findings of the present study demonstrate that the combination of NAT and tPA is a highly effective means of ameliorating BBB dysfunction, histological damage and functional deficits following stroke. Furthermore, NAT was able to counteract any negative consequences associated with tPA administration, such as an increased risk of haemorrhage and death. No other studies have previously investigated the combined use of an NK_1 receptor antagonist with a thrombolytic agent.

A role for tPA in BBB dysfunction following ischaemia has been widely documented (Kahles et al., 2005; Kelly et al., 2006; Pfefferkorn and Rosenberg, 2003; Yang et al., 2007; Yepes et al., 2003). As early as 1 h post-stroke, blood vessel associated tPA activity can be observed, followed by an increase in vascular permeability at 5 h post-MCAO (Yepes et al., 2003). In the present study, intravenous administration of tPA to naïve animals resulted in opening of the BBB. This was completely reversed by NAT treatment. EB is quite a large molecule (approximately 68 000 Da) and as such, passage of EB through the BBB requires considerable permeability changes. Increased BBB permeability has also been observed in uninjured animals following i.c.v administration of tPA (Yepes et al., 2003), with the degree of damage to the BBB correlated with the dose of tPA (Burggraf et al., 2007). Significant BBB permeability was still observed following administration of tPA at the onset of reperfusion. Subsequent combination of tPA with NAT reduced permeability to levels comparable to shams. The fact that NAT was able to reduce the permeability of the BBB in both naïve animals and stroke animals following tPA administration, suggests that the mechanism whereby tPA disrupts the BBB in both conditions may involve SP. This is consistent with the

findings of chapter 4, where increased SP levels within the infarcted hemisphere were associated with significant breakdown of the BBB and the formation of cerebral oedema, both of which could be attenuated by blocking SP binding with an NK$_1$ receptor antagonist.

One mechanism by which tPA may open the BBB is via low-density lipoprotein related receptor protein (LDL) (Benchenane et al., 2005; Makarova et al., 2003). LDL regulates vascular permeability in the brain under physiological and pathological conditions. It acts as a functional receptor for MMP-9 (Hahn-Dantona, 2001) and interactions between LRP and tPA are key regulators in the integrity of the neurovascular unit (Polavarapu et al., 2007). tPA-induced opening of the BBB (Cinelli et al., 2001; Yepes et al., 2003) can be ameliorated by an LRP antagonist but not by the NMDA receptor antagonist MK-801, suggesting that LRP is integral to tPA-induced barrier opening. Whilst the NMDA receptor was not integral to BBB opening, tPA may potentiate neurotoxicity via an interaction with the NR1 subunit of the NMDA receptor (Kaur et al., 2004). Cleavage of the NR1 subunit by tPA leads to NMDA receptor activation and the potentiation of excitotoxicity via Ca^{2+} currents, in addition to further tPA release from neurons (Wang et al., 1998).

An interaction with MMPs has also been proposed as a mechanism whereby tPA induces opening of the BBB (Pfefferkorn and Rosenberg, 2003; Horstmann et al., 2003; Wang et al., 2004; Kahles et al., 2005; Ning et al., 2006). Indeed, an increase in MMP-9 is observed in human (Montaner et al., 2003; Ning et al., 2006) and experimental (Aoki et al., 2002; Romanic et al., 1998; Sumii and Lo, 2002) stroke and is regarded as a negative prognostic factor (Ning et al., 2006). Such activation

of MMPs in the acute phase following stroke weakens vessel integrity by attacking the neurovascular matrix, increasing the risk of rupture and haemorrhagic transformation. Activation of MMPs has a direct effect on the integrity of the extracellular matrix and basal lamina, with integral structural proteins being degraded. Such disruption of the vascular architecture leads to increases in BBB permeability, oedema formation and haemorrhagic transformation. All of these events further exacerbate ischaemic damage and may lead to extension of the infarct. Thrombolysis in an embolic model of stroke was associated with significantly increased MMP-9 expression, accompanied by increased cerebrovascular permeability and vasogenic oedema (Lapchak et al., 2000). As such, a link between tPA therapy, MMP-9 expression and haemorrhagic transformation is well documented and may explain how tPA affects barrier integrity following stroke. Therefore, inhibition of MMPs may improve the safety profile for tPA.

Although an interaction between SP and MMPs in the brain has not been documented, a role in extracellular matrix metabolism has been reported in other tissues. In the lung, SP upregulates MMP-1 expression (Ramos et al., 2007) and significantly correlates with MMP-12 levels in chronic obstructive pulmonary disease (Xu et al., 2007). Also, SP can induce the secretion of MMP-2 from human synovial fibroblasts, whilst increasing overall MMP activity. Furthermore, another neuropeptide, VIP, has been reported to have a role in connective tissue metabolism, contributing to the inflammatory process of arthritis (Rahman et al., 1992). Insofar as SP may have a similar effect on MMPs within the brain as in peripheral tissues, an involvement of SP in the barrier dysfunction observed

following stroke is feasible. A reduction in MMP expression may potentially be a mechanism whereby NAT affords protection at the level of the BBB in the acute phase following stroke. These potential interactions requires further study, particularly since early disturbances in MMP expression are likely to be destructive whereas later changes in MMP expression may facilitate tissue remodelling and recovery (Asahi et al., 2000; Sood et al., 2007; Tejima et al., 2007).

In lung tissue infusion, of SP induces a dose-dependent increase in tPA release and activity, accompanied by increased blood flow (Newby et al., 1999; Newby et al., 2001). Although there are no published reports of an association between SP and tPA within the brain, it is tempting to speculate that similar events may occur in the brain as in peripheral tissue. Indeed, neurogenic inflammation has recently been demonstrated to occur brain injury, once thought only to occur in peripheral tissues (Donkin et al., 2007; Nimmo et al., 2004; Vink et al., 2003). Accordingly, the effect of SP on tPA and the plasminogen system requires further study. Similarly, neuroserpin is the endogenous inhibitor of tPA (Yepes and Lawrence, 2004) and a number of studies have reported neuroprotection following its administration in stroke (Cinelli et al., 2001; Yepes et al., 2000; Zhang et al., 2002). Direct injection of neuroserpin into the ischaemic area (Yepes et al., 2000) or over-expression of the neuroserpin gene (Cinelli et al., 2001) not only leads to a reduction in tPA activity but also a marked decrease in infarct volume in rat MCAO. Conversely, when the plasminogen system is interfered with a significant increase in infarct volume results. Such studies confirm the deleterious roles that tPA plays in ischaemia and the benefits of efficient thrombolysis.

In the current study, an increase in the incidence of ICH was observed in the tPA group, not surprising in light of the reports of increased risk of haemorrhage with tPA therapy. In a thromboembolic model of stroke, tPA treatment was associated with a 67% increase in the rate of haemorrhage (Lapchak, 2002), which is comparable with the present study. A 3-fold increase in haemorrhage was independently reported in tPA-treated versus untreated groups (Hacke, 1995). However, there has been one report of no effect of tPA treatment on mortality or haemorrhage (Toomey et al., 2002). In the present study, such an increase in ICH was associated with an increase in mortality within the tPA-treated group. Adjunctive treatment with NAT was able to reduce both the haemorrhage and mortality associated with tPA administration. Previous studies have also shown the efficacy of adjuvant treatment on reducing haemorrhage following tPA administration (Lapchak, 2002). A close association exists between blood brain barrier dysfunction and haemorrhagic transformation (Knight et al., 1998; Latour et al., 2004; Warach and Latour, 2004) and the mechanism whereby NAT/tPA was able to afford protection from ICH and death is most likely due to stabilisation of the BBB and the neurovascular unit. This would circumvent barrier disruption and haemorrhagic transformation. As an example, MMP-9 inhibition was found to reduce tPA-mediated mortality in cerebral ischaemia (Pfefferkorn and Rosenberg, 2003). Inhibition of proteolysis decreases the risk of haemorrhagic transformation following reperfusion, presumably via an MMP-related mechanism.

The present study is one of the first to extensively examine the effect of tPA administration on neurological outcome following stroke. Generally, tPA treatment was associated with a modest improvement in outcome, as compared to vehicle

treatment across the battery of functional outcome tests, although complete resolution of functional deficits was not observed. However, treatment with NAT, either alone or in combination with tPA, was effective in ameliorating functional deficits. The improvement in functional outcome observed in the tPA-treated group compared to the vehicle-treated group may in part be explained by the beneficial intravascular effects of tPA. The present study used a thread model of MCAO where occlusion is achieved through mechanical obstruction of the artery. During the period of occlusion a blot clot forms on the tip of the thread, which may be dislodged upon thread withdrawal and reperfusion. Administration of tPA at this stage would reduce the likelihood of any clots released during reperfusion, thereby maintaining the patency of the vasculature with a host of beneficial downstream effects. This may have also had favourable effects on BBB integrity, as reflected by the decreased EB extravasation in the tPA group compared to vehicles. A number of experimental studies have investigated the use of adjunctive agents, such as spin-trap agents, with tPA, and their effect on neurological deficits. In these studies, tPA had no effect on neurological deficits when administered alone, but when combined with an adjunctive therapy significant improvements in neurological function were observed (Bowes et al., 1995; Toomey et al., 2002; Zhang et al., 2003). Taken together, these studies demonstrate that adjunctive treatment with tPA is an effective means of reducing the functional deficits associated with stroke and improving outcome.

Previous studies have reported that tPA administration is associated with a worsened histological outcome (Wang et al., 1998; Yepes et al., 2000). However, this was not observed in the present study. Administration of tPA generally

produced a pattern of histological injury that was comparable to vehicles, although for some histological markers, a modest improvement was observed. Excess tPA in the CNS is associated with neuronal death (Wang et al., 1998), confirmed by the observation that injection of neuroserpin into the ischaemic zone decreases the number of apoptotic cells (Yepes et al., 2000). This may, in part, explain the lack of histological improvement observed in the tPA group. Normally, the presence of tPA leads to neutrophil accumulation and subsequent free radical production that can exacerbate injury (Asahi et al., 2000). In addition, SP may act as a chemotactic factor for monocytes (Ruff et al., 1985), leading to the accumulation of macrophages within the tissue. As such, the absence of plasminogen activators may retard neutrophil and macrophage migration into the ischaemic area (Siao and Tsirka, 2002). As these inflammatory cells are a major source of MMPs (Amantea et al., 2007) a decrease in their influx into the ischaemic tissue may reduce the degree of injury. This may have been one mechanism whereby NAT/tPA was able to convey some histological protection following stroke by counteracting some of the negative consequences of tPA therapy.

An increase in astrocyte hypertrophy and hyperplasia was observed following stroke in all treatment groups, but was most profound in the NAT treatment group, with a marked astrocyte response to injury. The main cell types involved in the formation of a glial scar are astrocytes, macrophages, microglia and oligodendrocytes. However, the final structure is predominately astrocytic. Research suggests that the astrocyte component of the glial scar may actively repair the BBB after injury. This is in keeping with a profound astrocyte response in the NAT treatment group, which was accompanied by maintenance of BBB integrity as reflected by reduced EB

extravasation. However, the role of astrocytes following injury may also encompass a deleterious component, as significant MMP-9 has been detected in microvessels and associated astrocytic end feet (Asahi et al., 2001). Exposure of cortical astrocytes to plasminogen activators leads to expression of inflammatory mediators such as MMPs, cytokines and chemokines (Lee et al., 2007), although inhibition of MMP-9 had no effect on glial scar formation (Copin and Gasche, 2007). Nevertheless, it is clear that plasminogen activators have pleiotropic actions beyond clot lysis.

An extensive macrophage response to infarction was observed in all treatment groups following stroke. However, it was most profound in the vehicle and tPA groups. Some studies have implicated a role for tPA in the modulation of the macrophage inflammatory response. Specifically, the absence of plasminogen activators reduces macrophage migration into brain tissue (Siao and Tsirka, 2002; Ling et al., 2006), tPA knockout mice demonstrate attenuated microglial activation (Gravanis and Tsirka, 2005) and PAI-1 deficient mice show defective macrophage migration (Cao et al., 2006). Therefore, attenuation of tPA neurotoxicity by NAT may have reduced macrophage influx into the infarct. Attenuation of SP function may also have further contributed to the reduced macrophage flux by inhibiting the action of SP as a chemotactic factor for monocytes.

Treatment with NAT/tPA provided the most profound reduction in axonal injury, as assessed at 7 d post-reperfusion. Whilst the exact mechanism whereby the combination therapy was able to afford some degree of white matter preservation is unknown, this may in part explain the improvements in functional outcome

observed in this group. Expression of APP by cortical neurons was largely unaffected by any of the drug treatments, suggesting a potential protective role for the neuronal APP changes that were observed. This is consistent with the improved functional outcome that has been recently reported following APP-α administration in a model of TBI (Thornton et al., 2006).

Treatment with tPA, NAT or NAT/tPA had no significant effect on infarct volume, despite a trend towards a decreased degree of infarction in the NAT/tPA group. Given the profound improvements in functional outcome observed, an improvement in lesion size was anticipated. However, a lack of improvement in lesion size associated with improved outcome has previously been reported following experimental stroke. In a thread occlusion model of stroke that reported no effect on lesion size following tPA administration despite improved outcome (Meng et al., 1999). The authors concluded that tPA did not appear to have any neurotoxic effects in models where reperfusion was guaranteed. In the present study, functional improvement despite a lack of reduction in lesion volume is likely to reflect synaptic plasticity of the peri-lesion area. As discussed in chapter 4, infarct volume may not be an accurate predictor of functional capacity. Indeed, previous studies have revealed decreased infarct volume after tPA administration at 2, 4 or 6 h following stroke with no amelioration of neurological deficits (Toomey et al., 2002). Similarly, reduced lesion volumes observed in tPA deficient mice and more extensive infarction in PAI-1 deficient mice following stroke have not correlated with functional outcome (Nagai et al., 1999). However, administration of neuroserpin decreased lesion volume and reduced the number of cells showing apoptotic features (Yepes et al., 2000). The sole report of the effect of a SP

antagonist on infarction reported a reduced infarct volume as assessed at 24 h stroke by TTC staining. Whilst the present study did not demonstrate a reduction in lesion size with tPA, the previous reports in the literature clearly support that under some conditions tPA has the capacity to affect lesion size and therefore outcome following stroke.

Other researchers have suggested that the window for thrombolysis may be extended beyond 3 h in certain cases (Kent et al., 2003). Indeed, findings of the DIAS trial revealed that the therapeutic window for thrombolysis may be extended up to 9 h following stroke onset (Hacke et al., 2005). The authors propose that modern imaging technology will enable identification of those individuals with a perfusion/diffusion mismatch who are amenable to later intervention without a reduction in treatment effect. However, not all patients would benefit from delayed treatment and future studies should focus on adjunctive therapies that make reperfusion safer and that extend the treatment window for all patients with acute ischaemic stroke.

5.5 Conclusions

The combination of tPA with NAT is a highly effective therapeutic intervention for ischaemic stroke. Not only does this intervention allow for the breakdown of blood clots impeding blood flow, it also reduces BBB permeability and histological injury and improves neurological outcome. The combined treatment approach permits the beneficial actions of tPA to occur whilst also reducing its' negative effects such as increased risk of haemorrhage, and potentially death.